U.58#4

The Journal of LABORATORY and CLINICAL MEDICINE

MATTHEWS LIBRARY
A.S.U. TEMPE, ARIZ.

The Official Publication of the Central Society for Clinical Research

Volume 58 | Number 4

October, 1961

WILLIAM H. DAUGHADAY, M.D., *Editor*
NEAL S. BRICKER, M.D., *Associate Editor*
St. Louis, Mo.

Board of editors

ROBERT H. FURMAN, M.D.
Oklahoma City, Okla.

ROBERT A. GOOD, M.D.
Minneapolis, Minn.

HARPER K. HELLEMS, M.D.
Jersey City, N. J.

ROBERT E. HODGES, M.D.
Iowa City, Iowa

ROBERT M. KARK, M.D.
Chicago, Ill.

RICHARD L. LANDAU, M.D.
Chicago, Ill.

GORDON MEIKLEJOHN, M.D.
Denver, Colo.

CHARLES A. OWEN, M.D.
Rochester, Minn.

OSCAR D. RATNOFF, M.D.
Cleveland, Ohio

ROBERT F. SCHILLING, M.D.
Madison, Wis.

MARVIN D. SIPERSTEIN, M.D.
Dallas, Texas

GENE H. STOLLERMAN, M.D.
Chicago, Ill.

rty-fourth annual meeting,
tral Society for Clinical
earch, November 3 and 4,
l, Drake Hotel, Chicago,
nois

E C. V. MOSBY COMPANY
blishers

Washington Blvd.
Louis 3, Missouri

OCT

DIFCO

ASTO

Difco reagents for the diagnosis of

Group A Streptococcal Infections

- RHEUMATIC FEVER
- GLOMERULONEPHRITIS

ANTISTREPTOLYSIN *O* TITERS (ASTO) and their relation to pathological conditions in Group A streptococcal infections have established the importance of this determination as a routine clinical test.

Bacto-Streptolysin O Reagent—a dehydrated, standardized and stable reagent requiring only rehydration with distilled water. Antistreptolysin *O* titers have been impractical for routine diagnosis because of the difficulties in preparing the reagent. Bacto-Streptolysin *O* Reagent is a standardized preparation permitting the routine performance of this diagnostic test in all clinical laboratories.

Bacto-ASTO Standard—an antiserum titered in Todd units for use as a control in the determination of antistreptolysin *O* titers.

Descriptive literature sent upon request

DIFCO LABORATORIES
DETROIT 1, MICHIGAN

MATTHEWS LIBRARY
A.S.U. TEMPE, ARIZ.

The Journal of
LABORATORY and CLINICAL MEDICINE

Volume 58, Number 4 OCTOBER 1961

Contents

Clinical and experimental

Effects of steroid-17-spirolactones on aldosterone secretion, excretion, and metabolism in man, 505

Eugene T. Davidson, M.D., William S. Coppage, Jr., M.D., Donald Island, B.S., and Grant W. Liddle, M.D., Nashville, Tenn.

Hormone production and nitrogen excretion in paralytic acute anterior poliomyelitis, 515

Bernt Hökfelt, M.D., and Ingmar Jungner, M.D., Stockholm, Sweden

Ultrafiltration studies on serum magnesium in normal and diseased states, 531

Ananda S. Prasad, M.B., B.S., Ph.D., Edmund B. Flink, M.D., Ph.D., and Robert McCollister, M.D., Minneapolis, Minn.

Changes in serum and spinal fluid calcium effected by disodium ethylenediaminetetraacetate, 542

Alfred Soffer, M.D., and Taft Toribara, Ph.D., Rochester, N. Y.

Lipid patterns in Niemann-Pick disease, 548

J. A. Balint, M.B., M.R.C.P., W. L. Nyhan, M.D., Ph.D., Paul Lietman, M.D., and D. A. Turner, Ph.D., Baltimore, Md.

A method for evaluation of disease and treatment in chronic leukemia, 559

Writing Committee: S. William Ross, M.D., Chairman, Eleanor J. MacDonald, B. A., Phil Davis, M.D., James Hammarsten, M.D., and William C. Levin, M.D.

Red cell transaminase levels in anemia. II. Thalassemia minor, 580

Martin D. Sass, M.S., and Paul W. Spear, M.D., New York, N. Y.

Contents continued on page 3

NEW Clot-Timer *For Highly Reproducible-Automatic* Prothrombin Times

The Clot-Timer operates on a simple principle. *For Plasma Clotting Times* — Gel-formation representing end-point, changes both viscosity and surface tension of reaction mixture. During reaction, rotor arms pass through solution in reaction cups, draining clean and passing two electrodes by a precise clearance. When fibrin gel begins forming, rotor arm instantly picks up a drop of the reaction mixture, transfers the drop to electrodes. This closes the detector circuit, stopping digital timer which was started when reaction was initiated. The principle for whole blood clotting times is similar, except the first fibrin thread to be picked up from drop of blood closes the circuit.

Highly reproducible results; speed; simplicity; and reliability because of high quality long-life construction are characteristic of the Clot-Timer.

For complete information on the NEW CLOT-TIMER, write for Bulletin CT-8.

Aloe #10962 CLOT-TIMER, Model 201: complete instrument, including 12 each of reaction cups, reaction rotors, blood plates and blood rotors.

aloe scientific
DIVISION OF BRUNSWICK CORPORATION
General Offices: 1831 Olive St. • St. Louis 3, Missouri

FULLY STOCKED DIVISIONS COAST-TO-COAST

Contents *continued*

Red cell transaminase levels in anemia. III. Acute and chronic blood loss, 586

Martin D. Sass, M.S., and Paul W. Spear, M.D., New York, N. Y.

Catalase activity and redox activity in platelets of patients with rheumatoid and nonrheumatoid inflammatory states, 592

Grace P. Kerby, M.D., and S. M. Taylor, B.A., Durham, N. C.

The use of I^{131}-labeled contrast media in scintillation scanning of the kidney, 598

Thomas P. Haynie, M.D., Mohammed M. Nofal, M.D., Edward A. Carr, Jr., M.D., and William H. Beierwaltes, M.D., Ann Arbor, Mich.

The comparison of the apparent volume of distribution of large molecular weight dextran and Evans blue dye, 605

Walter Lyon Bloom, M.D., Atlanta, Ga.

Clubbing of the digits: Physiologic considerations, 613

F. A. Bashour, M.D., Ph.D., Dallas, Texas

Transport of vitamin A across human intestine in vitro, 622

M. R. Loran, Ph.D., T. L. Althausen, M.D., F. W. Spicer, M.D., and W. I. Goldstein, B.S., San Francisco, Calif.

Effect of amino acids on growth and susceptibility to viral hepatitis in mice, 627

Boris H. Ruebner, M.D., and Katsumi Miyai, M.D., Baltimore, Md.

A comparative study of monkey kidney cell cultures and suckling mice for the recovery of enteroviruses from patients with central nervous system disease, 634

Edwin H. Lennette, M.D., Ph.D., Anna Wiener, B.S., Isao Hoshiwara, M.D., James Woodie, M.S., and Robert L. Magoffin, M.D., Berkeley, Calif.

Plasma protein antigens in the histopathologic vascular response to experimental sensitization, 644

Sheldon G. Cohen, M.D., and Theresa M. Sapp, M.T., Wilkes-Barre, Pa.

Contents continued on page 5

for the diarrheal attack

effective–eradicates enteric bacterial pathogens

selective–does not eradicate the normal intestinal flora[1]

FUROXONE® LIQUID

brand of furazolidone

New, convenient prescription size: bottle of 2 oz. Also: bottle of 16 oz.

■ Exceptionally broad bactericidal range includes species and strains now resistant to other antimicrobials ■ Virtually nontoxic ■ Does not encourage monilial or staphylococcal overgrowth ■ Has not induced significant bacterial resistance ■ Dosage may be found in your PDR.

FUROXONE LIQUID is a pleasant orange-mint flavored suspension containing FUROXONE 50 mg. per 15 cc., with kaolin and pectin.

1. Mintz, A. A.: Antibiot. Med. 7:481, 1960.

EATON LABORATORIES, Division of The Norwich Pharmacal Company, NORWICH, N. Y.

Contents *continued*

Experimental studies in the anemia of malignancy. I. The antiglobulin test in tumor-bearing hamsters, 652

Anthony Betts, M.D., Perry G. Rigby, M.D., Charles P. Emerson, M.D., and Gilbert H. Friedell, M.D., Boston, Mass.

Laboratory methods

The use of radioisotopes to measure body fluid constituents. I. Plasma sulfate, 656

E. Miller, M.S., C. J. Hlad, Jr., M.S., Stanley Levine, J. H. Holmes, M.D., and H. Elrick, M.D., Denver, Colo.

Determination of phenformin in biologic fluids and tissues, 662

Louis Freedman, Ph.D., Milton Blitz, B.S., Ephraim Gunsberg, B.S., and Sylvia Zak, B.S., Yonkers, N. Y.

A colorimetric dry weight determination for tissue cultures and tissue homogenates, 667

J. Martyn Bailey, Ph.D., and A. Meymandi-Nejad, B.Sc., Washington, D. C.

Vol. 58, No. 4, October, 1961. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States and its Possessions $13.00; Canada, Latin America, and Spain $14.00; Other Countries $15.00. Students, interns, and resident physicians: United States and its Possessions $7.80; Canada, Latin America, and Spain $8.80; Other Countries $9.80. Single copies $2.00 postpaid. Second class postage paid at St. Louis, Mo. Printed in the U. S. A. Copyright © 1961 by The C. V. Mosby Company.

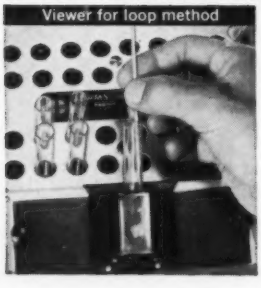
now! "PRO-TIMES" with split-second accuracy...

(Pat. No. 2,932,718)

ADAMS **THROMBITRON** T.M.

assures reproducible results using standard technics

In prothrombin time determinations highly accurate, reproducible results are essential; with the new Adams Thrombitron they are certain. The sensitive mercury thermostat ensures constant, dry 37°C (±0.5°) temperature of plasma, thromboplastin, and prothrombin pipettes. The Thrombitron eliminates the need for water bath. With the Thrombitron, all the required test components are within finger tip reach. The entire test can be performed with the technologist seated at the lab table—no wasted time or motion.


Viewer for loop method

Unit is designed for either accepted standard method—"tilt" or "loop." A constant light source and magnifying viewer assure accurate observation. The Adams Thrombitron has no moving parts or complex electrical circuits — no maintenance problems. Price **$225**

AVAILABLE FROM YOUR DEALER
For further information write to

Clay-Adams New York 10, N. Y.

The Journal of
LABORATORY and CLINICAL MEDICINE

Published monthly under the editorial direction of a Board of Editors appointed by the Central Society for Clinical Research. Neither the Board of Editors nor the Publisher accepts responsibility for the statements made by contributors.

Editorial communications: Correspondence relating to the publication of manuscripts should be addressed to the editor.

MANUSCRIPTS:

Scope and selection. The Journal welcomes the submission of original manuscripts describing investigations in the broad fields of laboratory and clinical medicine. Papers submitted by nonmembers of The Central Society for Clinical Research will be given equal consideration. Reports of purely laboratory or animal investigations should have a clear bearing on the problems of human disease. The section on Laboratory Methods is reserved for new methods or significant modifications of important existing methods.

Organization. The original and one copy of the manuscript should be submitted. Manuscripts should be double spaced on one side of the paper. The following organization is recommended: 1. Abstract (250 words or less orienting the problem, describing the observations, and stating the principal conclusions). 2. Introduction. 3. Methods. 4. Results. 5. Discussion. Unusual abbreviations should be avoided and standard chemical or nonproprietary pharmaceutical nomenclature employed.

References. Bibliographic references should conform to the current style of the Journal and include: Authors, Title, Journal, Volume, Page, and Year. It is the author's responsibility carefully to verify each reference.

Illustrations. Results may be presented in tables or figures, but the same data may not be presented in both tables and figures. A reasonable number of illustrations are permitted without extra charge. Arrangements for the use of color plates or the reproduction of figures requiring special engraving or paper may be made with the Editor. Glossy photographic prints in which the details are clearly evident or original illustrations on good quality paper in which the lining and lettering are done with India ink or black typewriter ribbon must be provided for the reproduction of figures.

WILLIAM H. DAUGHADAY, M.D. *Editor*
NEAL S. BRICKER, M.D. *Associate Editor*
Department of Medicine, Washington University School of Medicine
600 S. Kingshighway, St. Louis 10, Mo.

Business communications. All communications in regard to advertising, subscriptions, change of address, etc., should be addressed to the Publishers.

Subscription rates. United States and its Possessions $13.00; Canada, Latin America, and Spain $14.00; Other Countries $15.00. Students, interns, and resident physicians: United States and its Possessions $7.80; Canada, Latin America, and Spain $8.80; Other Countries $9.80. Single copies $2.00 postpaid. Remittances should be made by check, draft, post office or express money order, payable to this Journal.

Publication order. The monthly issues of this Journal form two semiannual volumes; the index is in the last issue of the volume—in the June and December issues.

Change of address notice. Six weeks' notice is required to effect a change of address. Kindly give the exact name under which a subscription is entered, and the full form of both old and new address, including the post office zone number.

Advertisements. Only articles of known scientific value will be given space. Forms close first day of the month preceding date of issue. Advertising rates and page sizes will be furnished on application.

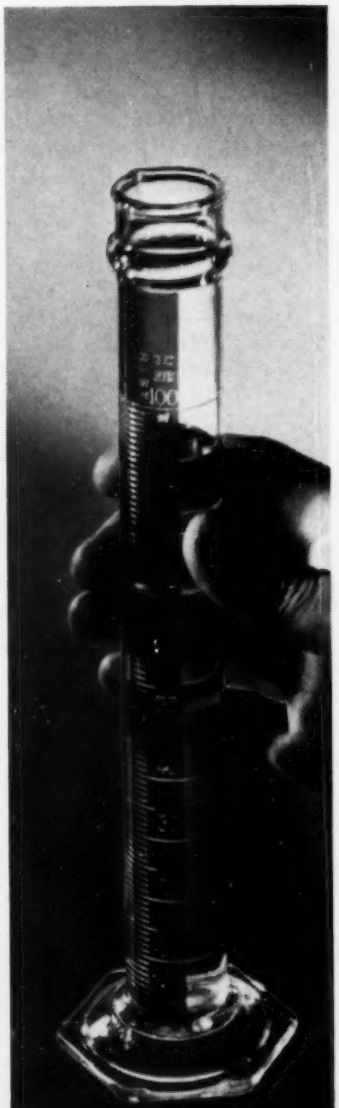
Reprints. Reprints must be ordered directly from the Publishers, who will send their schedule of prices. Individual reprints must be obtained through the author.

Bound volumes. Publishers' Authorized Bindery Service, 430 West Erie Street, Chicago 10, Illinois, will quote prices for binding complete volumes in buckram.

THE C. V. MOSBY COMPANY, *Publisher*
3207 Washington Blvd., St. Louis 3, Mo.
Published monthly. Subscriptions may begin at any time.

HOW IMPORTANT IS PRICE

When you look at prices on cover slips, consider the special dispenser you get with CORNING cover glass. The glass is clean; often you can use it right from the box. It also makes for cleaner, faster handling.

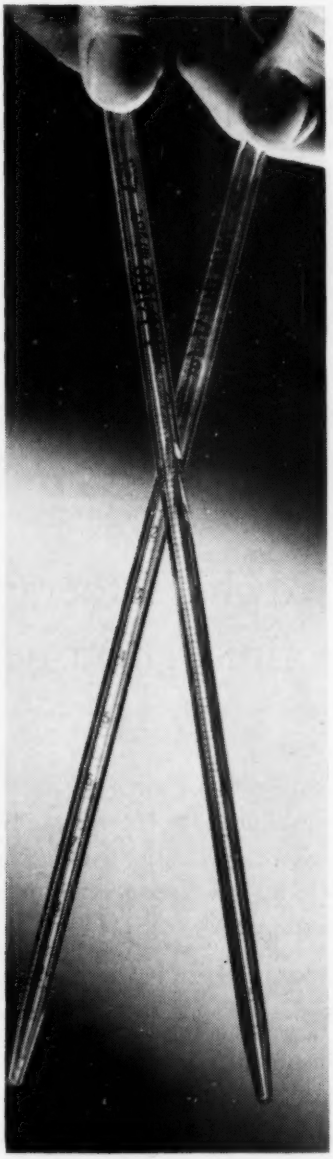


When you look at prices on graduated cylinders, consider the extra strength you get with PYREX graduates. That shoulder under the rim takes the shock of an upset, prevents the rim's striking the bench. The markings are permanent. They're etched through a layer of red glass.

When you look at the prices on pipets, consider the extra life of the markings on our ACCU-RED pipets. The marks are built into the glass, last as long as the pipet. Now you can get them color coded at no extra cost.

N BUYING LABWARE?

hen you look at prices on ntrifuge tubes, consider e extra strength built into YREX tubes. The ends are ccurately formed and uni- rm to take the strain of ntrifuging. The permanent aduations of this ACCU- ED model are applied with- t etch marks in the glass, hich often weaken centri- ge tubes.



When you look at the prices on pipets, consider the extra life of the markings on our ACCU-RED pipets. The marks are built into the glass, last as long as the pipet. There are no etch marks to weaken the glass. No fillers to wear off or fall out.

It's an unfortunate fact that a purchaser cannot determine the *cost* of labware simply by holding it up against a scale of *prices*.

Check your own experience and you'll find that, although two brands of the same item may be *priced* alike, one will outlast or be easier to use than the other, and actually *cost* you less.

You'll find that we build into PYREX® labware useful features that make it cost you less than labware priced the same or even lower. For example: pipet tips have two bevels to eliminate rough edges which cause breakage; graduate tops have reinforcing beads to soak up impacts; a new dispenser pack for cover slips keeps them clean, ready for use.

Ask your laboratory supply representative to show you how to ferret out the actual *cost* of labware from the bewildering array of brands and *prices* you have to cope with.

CORNING GLASS WORKS

8210 Crystal St. • Corning, New York

CORNING MEANS RESEARCH IN GLASS

YREX® Laboratory Ware . . . *the tested tool of modern research*

clinical experience continues to indicate value of the CYTOTOXIC AGENT...

CYTOXAN®

Cyclophosphamide, Mead Johnson

for palliative chemotherapy of certain types of malignant neoplasms

"Cyclophosphamide [Cytoxan] has proved a valuable addition to chemotherapeutic drugs available for the treatment of malignant diseases of the haemopoietic and reticuloendothelial systems.... Particularly effective in Hodgkin's disease, lymphosarcoma, chronic lymphocytic leukaemia, and myelomatosis...."[1]

"Objective data suggest that this agent [Cytoxan] has advantages not possessed by standard alkylating agents now in clinical use."[2]

"With the use of cyclophosphamide [Cytoxan] there is a relative lack of thrombocytopenia and a diminution in gastrointestinal side-effects, so that it may offer therapeutic advantages over other alkylating agents."[3]

Other Advantages in Clinical Practice: Broad-spectrum application. High therapeutic index. No vesicant activity—may be given orally or parenterally.

(1) Matthias, J. Q.; Misiewicz, J. J., and Scott, R. B.: Brit. M. J. *2*:1837-1840 (Dec. 24) 1960.
(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage—The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

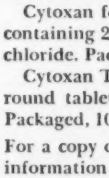
AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.



Mead Johnson Laboratories

Symbol of service in medicine

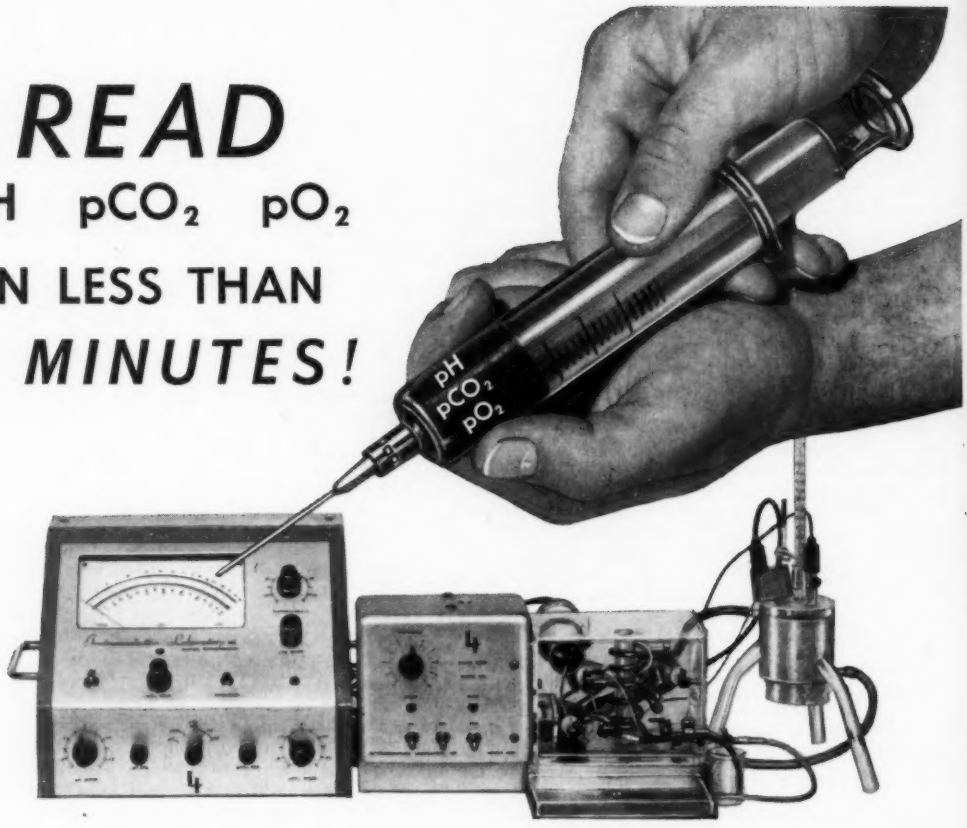
52361

READ

pH pCO_2 pO_2

IN LESS THAN

3 MINUTES!



with one volume of blood

simply • accurately • reliably

The IL-113 is a complete blood gas analyzing system designed for rapid, accurate measurement of the pH, pCO_2, and pO_2 of blood. The entire system . . . meter, bath and electrodes . . . requires a minimum of space with all controls conveniently located for ease and simplicity of operation.

The IL-113 maintains the highest degree of accuracy and reliability. It has a range of 6.8 to 8.0 pH, 10 to 100 mm Hg pCO_2 and 0 to 800 mm Hg O_2. The system is equipped with a pH electrode, pCO_2 electrode and pO_2 electrode. When used with these electrodes the overall precision of the system is greater than $\pm$.005 pH, $\pm$0.5 mm Hg pCO_2 and 1% pO_2.

The IL-113 measures all three parameters in less than three minutes. And it needs only 1.5 ccs. of blood for all three readings.

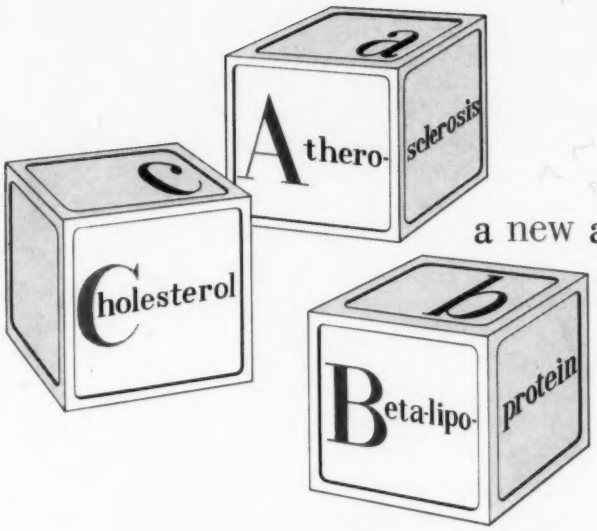
The IL-113 system comes complete with meter, constant temperature bath, electrodes, electrolyte, buffer, expendable supplies, installation and instruction anywhere in the U. S.

OVER 150 SYSTEMS IN CURRENT OPERATION —

FOR FURTHER INFORMATION — CALL OR WRITE

INSTRUMENTATION LABORATORY, INC.

108 CUMMINGTON STREET BOSTON 15, MASS., CO 6-5535



a new approach to these A-B-C's

A recent review of the **atherosclerosis** problem[1] points out that **"cholesterol** is only one of the serum lipids which may be related in some way to atherogenesis" and suggests that "specific triglycerides, phospholipids, fatty acids or the whole spectrum of **beta-lipoproteins** may be of equal or greater importance."

The role of the low-density beta-lipoproteins has been stressed by Olson[2], who suggests that they are the primary agents in atherosclerosis. Another recent discussion[3] has brought out the need for a simple and reliable testing procedure for beta-lipoproteins.

BETA-L TEST® is a specific immunochemical test for estimating serum beta-lipoprotein levels. BETA-L TEST reagent contains antibodies which specifically precipitate the beta-lipoproteins in human sera. This precipitate is then measured visually. Test requires only one drop of patient's serum and can be performed in 10 minutes. Clinical laboratories equipped with a micro-hematocrit centrifuge need no other special equipment for test performance.

In addition to its usefulness in atherosclerotic studies, BETA-L TEST may be found a helpful diagnostic and prognostic procedure in diabetes, hypothyroidism, the menopausal state and other conditions associated with elevated beta-lipoproteins.

BETA-L TEST measurements correlate well with total beta (low-density) lipoprotein measurements obtained by electrophoresis[4] and polyanion precipitation[4] and with cholesterol values in the beta-lipoproteins[5].

BETA-L TEST is supplied in 60-test kits.

1. Goldsmith, G. A.: Highlights on the Cholesterol-Fats, Diets and Atherosclerosis Problem, J.A.M.A. *176:* 783-790 (June 3) 1961.

2. Olson, R. E.: Prevention and Control of Chronic Disease. I. Cardiovascular Disease—with Particular Attention to Atherosclerosis, Amer. J. Public Health *49:* 1120-1128 (Sept.) 1959.

3. Wood, F. C., Gurin, S., and Kuo, P. T.: Medical Correlation Clinic on Atherosclerosis and Coronary Artery Disease, Am. Pract.-Dig. Treat. *12:* 235-247 (April) 1961.

4. Heiskell, C. L., et al.: A Simple Method for Quantitation of Serum Beta-Lipoproteins by Means of the Immunocrit, Amer. J. Clin. Path. *35:* 222-226 (March) 1961.

5. Bergquist, L. M., Carroll, V. P., Jr., and Searcy, R. L.: Evaluation of a Specific Antiserum for Serum-β-Lipoprotein Estimations, Lancet *i:* 537-538 (March 11) 1961.

HYLAND LABORATORIES
4501 Colorado Blvd., Los Angeles 39, Calif.
Branch Office: 160 Lockwood Ave., Yonkers, New York

Hyland

> ...from a metabolic point of view the young of different mammals have more in common with each other than with their parents. In fact, to look upon the young as a miniature adult could not be farther from the truth.[1]

The newborn is not a small adult

Physiologically and biochemically, the infant is a unique creature with very special characteristics. Just after birth and during the first week of life, adjustments to living apart from the maternal bloodstream result in blood chemistry patterns very different from those which are normal for adults.

Because of these differences, standards suitable for use in adult blood chemistry studies afford less assurance in determinations on the newborn, including the sometimes critically important bilirubin level.

You now have available a new Versatol standard-in-serum especially standardized for blood chemistry determinations in the newborn:

Versatol® Pediatric

Versatol® Pediatric

In Versatol Pediatric, all constituents are present in concentrations normal for serum of infants between birth and the fifth day of life—with one exception: bilirubin.

An elevated bilirubin standard. A great many pediatric laboratories have expressed a growing need for a Versatol standard-in-serum incorporating bilirubin at the critical 20 mg./100 ml. level. VERSATOL PEDIATRIC now fills that need, with bilirubin standardized at 20 mg. per 100 ml. in serum otherwise normal for the newborn. VERSATOL PEDIATRIC is the result of more than two years of coordinated investigation in over a dozen major university medical centers. A report on these studies was recently delivered before the Academy of Pediatrics Subcommittee on Bilirubin.

An alkaline phosphatase control. The alkaline phosphatase enzyme in VERSATOL PEDIATRIC has been replaced quantitatively to produce an exact amount of enzyme activity. Values for this activity, **based on recoveries,** are provided for the five most widely used methods. They are expressed in traditional units as well as International Units.

In adult liver function studies, VERSATOL PEDIATRIC is a new and valuable tool, providing a reference standard for elevated (for adults) alkaline phosphatase and elevated bilirubin.

VERSATOL PEDIATRIC, like all the Versatol series, is manufactured by the unique General Diagnostics process of selective dialysis, purification and weighing-back-in of serum constituents. Especially adaptable to micro- and semi-microchemical procedures, VERSATOL PEDIATRIC is packaged in vials to be reconstituted with 1.0 ml. of distilled water, 10 vials per box, $12.00 per box.

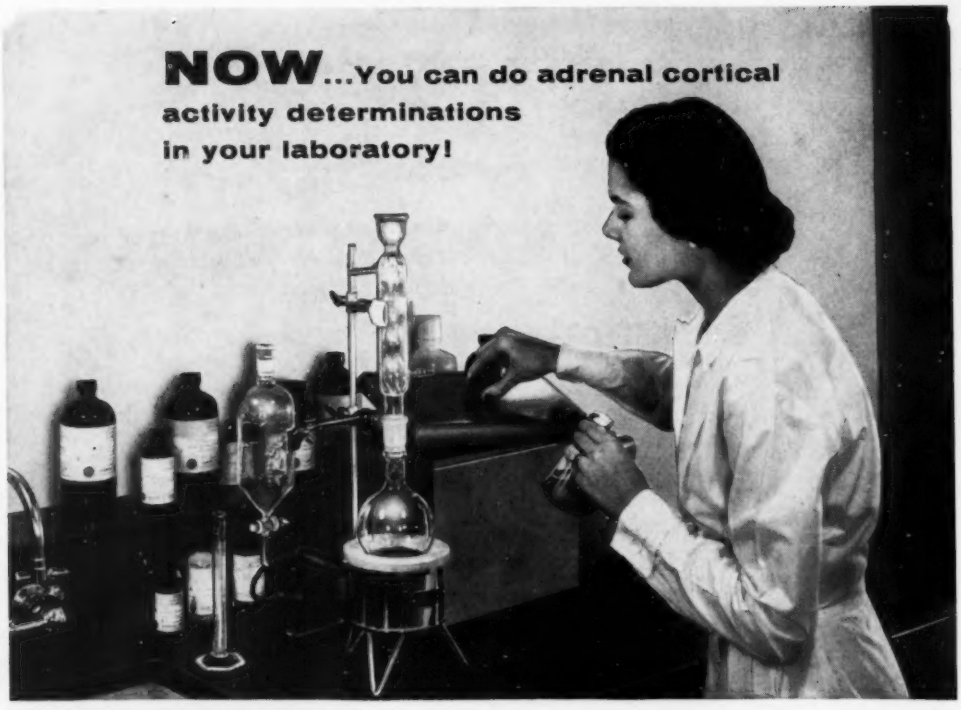
Remember: the Versatols are your only standards-in-serum with weighed-in constituents.

[1]Lathe, G. H. (Sass-Kortsak, Andrew, ed.) in **Kernicterus;** report based on symposium held at IX International Congress of Paediatrics, Montreal, July, 1959. University of Toronto Press, Toronto, 1961.

For maximum assurance in blood studies on the newborn, use the new infant-standardized Versatol Pediatric. Your distributor can supply you today.

GENERAL DIAGNOSTICS DIVISION

WARNER-CHILCOTT DIV. MORRIS PLAINS, N.J.



KETOSET

by **Hycel**

All these determinations are possible with the Ketoset—

- total neutral 17-ketosteroids
- 17-ketogenic steroids
- 17-reduced ketogenic steroids
- alpha-beta fractionations of the 17-ketosteroids
- qualitative dehydroisoandrosterone

For complete details consult your S/P representative or write Technical Service Department, Scientific Products, 1210 Leon Place, Evanston, Illinois.

In one integrated set, the Ketoset supplies 5 highly accurate laboratory determinations instrumental in differentiation of hypo-adrenal, normal-adrenal and hyper-adrenal cortical activity. In addition, hypo-activity may be differentiated into primary and secondary states; in case of hyper activity, carcinoma is readily distinguishable from hyperplasia or adenoma.

Procedure consists of pretreatment, hydrolysis, extraction, evaporation and color development. Time-saving, too—in as little as 1½ hours, a 17-ketosteroid determination can be completed. Ketoset consists of glassware, heaters, all reagents necessary for determination of 200 tests in various combinations and Vapo-Vent for evaporation of volatile, inflammable fluids.

No. 53853A—Hycel Ketoset, complete with Vapo-Vent, glassware, 2 heaters, necessary reagents for 200 tests in various combinations and complete instructions for use, without support stand or clamps.....**$459.00**

No. 53857—Extra Reagent Set—for 200 tests. each...**$169.00**

S/P

Scientific Products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

GENERAL OFFICES—1210 LEON PLACE, EVANSTON, ILLINOIS

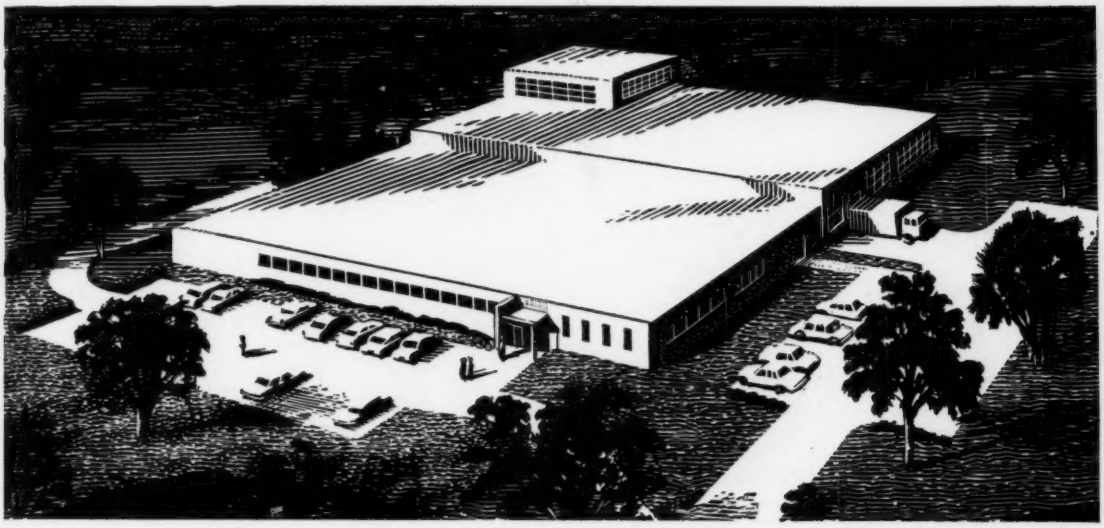
Regional Offices: Atlanta • Boston • Chicago • Columbus • Dallas • Kansas City • Los Angeles Miami • Minneapolis • New York • San Francisco • Washington

Export Department—Flushing 58, L. I., New York. In Canada: Canadian Laboratory Supplies Limited. In Mexico: Hoffmann-Pinther & Bosworth, S. A.

We interrupt our series to bring you a special announcement

IT HAPPENED THIS MONTH...

a glance at tomorrow in relation to today



IN OCTOBER – (1961) – Schwarz BioResearch dedicates this new building at Orangeburg, New York. It rededicates itself to providing the finest biochemicals, through integrated research, production, quality control, and distribution.

The new 30,000 sq. ft. building – housing an enlarged scientific staff and modern equipment – is designed to be a most efficient and most economical source of biochemicals. Thus, we are better prepared to meet your requirements, from the most exacting microscale radiochemicals for fundamental research to tonnage lots of compounds for industrial use.

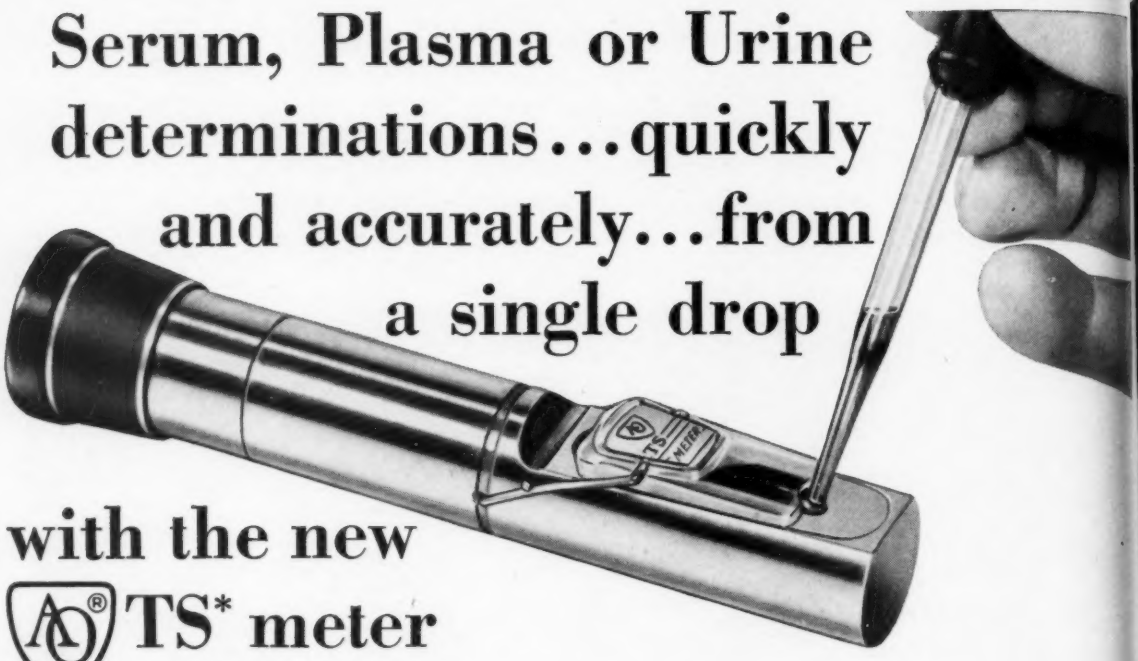
IN EVERY MONTH: *NEW BIOCHEMICALS—more nucleic acid compounds, intermediates in carbohydrate metabolism, peptides, and sulfhydryl compounds.—NEW RADIOCHEMICALS—more C^{14}-labeled and tritiated compounds—many at specific activity levels higher than heretofore obtainable—providing improved tools for precise studies of metabolic processes, and physiological and biochemical reactions.—NEW PHARMACEUTICALS—we anticipate an increasing need to prepare special types of biochemical compounds for use in clinical investigation. Such compounds may also be radiolabeled.—NEW STANDARDS OF PURITY—We accept the criteria of the National Research Council, recommended as a floor—not a ceiling—for our quality standards. More effective methods of production will allow even more exacting standards of purity. Each product you order will be accompanied by full and exact analytical data.—EXPANDED RESEARCH AND DEVELOPMENT—we will continue to provide an ever wider range of compounds for research in the structure and function of DNA and RNA. Our new facilities will permit us to do more contract research for government and industry. And we are expanding our development and manufacturing program for THIOGEL®, the new thiolated gelatin with special applications in the fields of enzyme stabilization, photography, drugs, and cosmetics.—SPECIAL SERVICES—facilities for custom preparation of labeled compounds will be substantially increased, especially in respect to tritiation and P^{32} incorporation.*

You are cordially invited to visit us at our new location. You are also invited to write for a copy of our new catalog.

SCHWARZ BIORESEARCH, INC. • Dept. 10K • Mountain View Ave., Orangeburg, N.Y.

BIOCHEMICALS • RADIOCHEMICALS • PHARMACEUTICALS *for research, for medicine, for industry*

Serum, Plasma or Urine determinations...quickly and accurately...from a single drop



with the new AO® TS* meter

(a Goldberg Refractometer)

The new AO TS Meter requires just *one drop* of sample to provide immediate, accurate determinations of total solids, specific gravity, refractive index, protein concentration or water concentration in serum, plasma and urine.

Minute Sample Required: As little as 0.02 ml of sample is sufficient for the most precise determination. Minute sample previously reported "q.n.s." now can be read routinely for example, in pediatric urinalysis, in oliguric patients, and in collection from ureteral catheters or microhematocrit tubes.

Temperature-Compensated for Unsurpassed Accuracy: The AO TS Meter provides automatic temperature compensation for all aqueous solutions. Differences in room, instrument or sample temperatures do not affect readings. Error from these sources is reduced to less than 0.1% over the range of 65° to 95°F. Readings are consistently obtained with a degree of accuracy, speed and reproducibility significantly better than those obtained with urinometers, the falling-drop method or with instruments uncompensated for temperature.

Direct Readings: You make an immediate analysis directly from the built-in scale . . . without references to graphs, tables or thermometer readings. Two models are available. Model 10400 provides determinations of total solids in plasma or serum and urine specific gravity; model 10401 gives readings of protein concentration and refractive index.

***Total Solids Meter.** The AO TS Meter and this method of analysis was developed in close cooperation with Dr. A. V. Wolf, Head of the Department of Physiology, University of Illinois, College of Medicine, Chicago, Ill.

Write for complete information!

American AO® Optical
COMPANY
INSTRUMENT DIVISION, BUFFALO 15, NEW YORK

Dept. X15

Gentlemen: Please send Brochure SB10400-with complete information about the new AO TS Meter.

Name .....

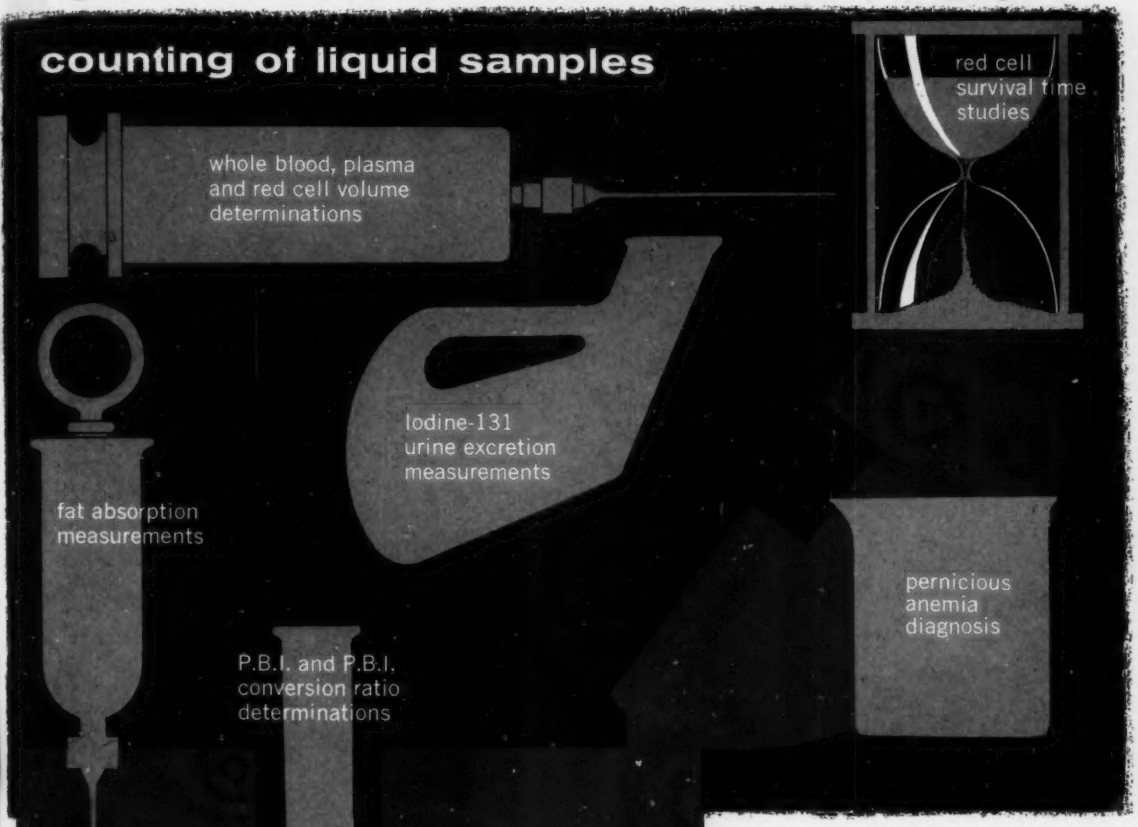
Address .....

City ..... Zone ..... State .....

IN CANADA write — American Optical Company Canada Ltd., Box 40, Terminal A, Toronto, Ontario

ways PICKER NUCLEAR can help you

counting of liquid samples


red cell survival time studies
whole blood, plasma and red cell volume determinations
Iodine-131 urine excretion measurements
fat absorption measurements
pernicious anemia diagnosis
P.B.I. and P.B.I. conversion ratio determinations

Blood, urine, saliva radioactivity measurements are made with greater ease and efficiency on a Picker well-type Scintillation Detector because . . .

- *it uses a larger crystal with 1" D well (holds samples up to 15 cc)*
- *it provides removable splash guards in the well or on top of the crystal*
- *it has 2" of lead shielding on all sides, including the bottom*
- *its fingerlift lid is counter-balanced*

The Picker well-type Scintillation Detector (Well Counter) is one of the comprehensive Picker line of nuclear instrumentation and supplies: all marketed and serviced through a national network of company offices staffed by trained Picker people. *Picker alone in the nuclear field offers this caliber of local service.*

For details call your district office (see 'phone book) or write Picker X-Ray Corporation, White Plains, New York.

Picker nuclear

Now you can monitor 2 events at once
. . . display a third on a panel meter . . .
change amplifiers as phenomena require

with this single compact, economical SANBORN DUAL MONITOR
specially designed for operating and recovery rooms

This new Sanborn 2-channel visual monitoring system is specifically designed for both large and small hospital use, where an easily operated, dependable and yet economical instrument is needed for 2-channel display of dynamic phenomena such as ECG, EEG, and blood pressure during or after surgery. Compact (12″ x 13½″ x 21″) and completely self-contained, the Model 762 Dual Monitor presents two sharp, clear traces simultaneously on a long-persistence 5″ cathode ray tube, with simple controls for sweep speed, screen scale illumination, brightness, and focus.

Your choice of *interchangeable, plug-in preamplifiers* equip the Dual Monitor for the phenomena to be displayed: ECG, EEG, and pressure types for 'scope display of dynamic events . . . Low Level and Carrier types for presentation of temperature, mean blood pressure, and other slowly changing events on a large scale, external "760" Monitor Meter unit available as an accessory. Also a cardiotachometer (760-3) can be used for indicating heart rate on the "760" Monitor Meter.

Safety in the presence of explosive gases is the reason all "760" preamplifiers are transistorized, low voltage units. The power supply has both current and voltage limiting circuits — eliminating the possibility of high voltage or current coming *from* the preamplifier *to the patient.* Installing the Dual Monitor five feet above floor level then affords complete protection.

Write for complete information on the versatile Model 762 Dual Monitor, interchangeable Preamplifiers, optional Amplifier-Meter unit and Recording Systems for use with it. Address inquiry to Manager, Research Instrument Sales.

MEDICAL DIVISION
SANBORN COMPANY
175 Wyman St., Waltham 54, Massachusetts

Cappel

DIAGNOSTIC ANTIGENS and ANTISERA

Other Cappel
Antigens and Antisera

ESCHERICHIA COLI ANTISERA

STREPTOCOCCUS GROUPING SERA
AND GROUP EXTRACTS

SALMONELLA ANTISERA
AND TYPING SERA

SHIGELLA GROUPING ANTISERA

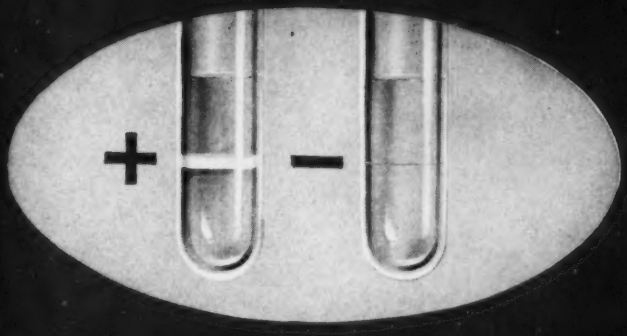
FEBRILE ANTIGENS

KLEBSIELLA TYPING SERA

C-REACTIVE PROTEIN
TEST REAGENTS

For demonstrating the
Neufeld Quellung Reaction—

**PNEUMOCOCCUS
TEACHING KIT**



PRECIPITIN SERA and
its NORMAL CONTROL SERA

GAMMA GLOBULIN ANTISERA

WHOLE SERUM ANTISERA

These stable, desiccated sera restore to a sparkling, crystal-clear solution that indicates purity and facilitates accurate test interpretation.

No other source supplies sera and antisera from so many species:

BOVINE	DOG	MOUSE
CAT	GOAT	RAT
CHICKEN	HAMSTER	RABBIT
DEER	HORSE	SWINE
	HUMAN	

Order today from your dealer . . . write for new Cappel Catalog and Price List.

CL

Cappel Laboratories

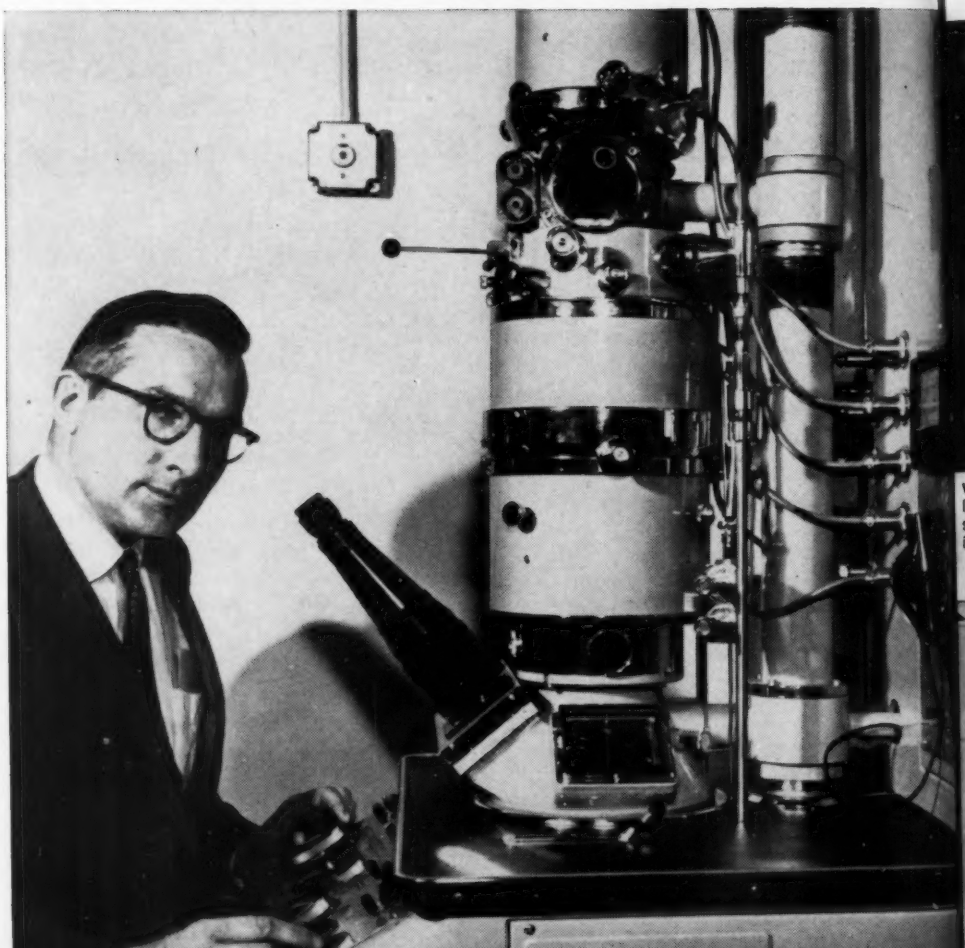
ANIMAL BLOOD PRODUCTS • TISSUE CULTURE REAGENTS • DIAGNOSTIC REAGENTS

West Chester, Pennsylvania

DIVISION OF B-D LABORATORIES, INC.

AGENTS: *Becton, Dickinson & Co., Canada, Ltd., Toronto 10, Ont.* FOR EXPORT: *Becton, Dickinson & Co., S.A., Colon, Panama*

FISHER SERVICES YOUR LA

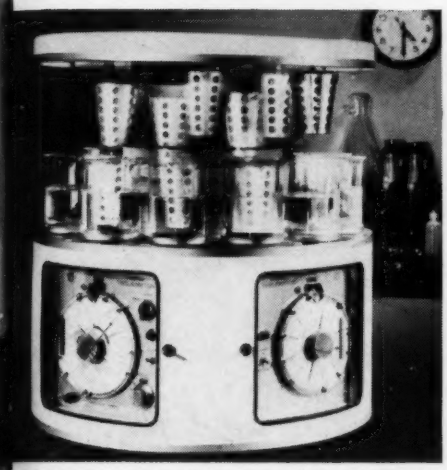


With 8A resolution, the JEM-6C Electron Microscope provides highest available resolving power fo biological and medical work. Direct magnification is continuously variable from 600X to 200,000X . . accelerating voltages are extremely stable . . . specimen change is fast. This Fisher Clinical Specialis specializes in the JEOL 'scopes, is ready to give on-the-spot help in their installation and use.

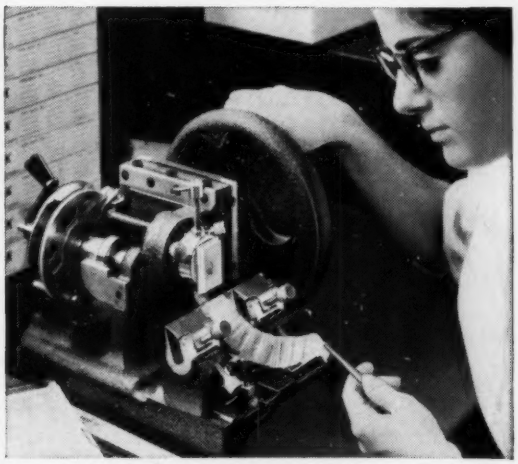
UR LAB NEEDS!

CHECKING a new JEOL Electron Microscope installed at Massachusetts General Hospital is a Fisher representative (left). He is one of Fisher's Clinical Specialists who demonstrate new instruments, explain efficient operating methods, trouble-shoot problems. This technical help . . . aid in lab layout and furniture planning . . . custom glass blowing . . . expert instrument repairs . . . are among the many services Fisher offers you.

Service is one part of the Fisher spectrum . . . others are indicated below. Summed up, they are the reason Fisher Scientific is a leader in laboratory instrumentation and reagent manufacture . . . and your **comprehensive source** for laboratory needs.



While you're away during day, night or weekend, Dual-Unit Tissuematon® automatically processes or stains dozens of specimens. It's another of the 787 instruments and appliances developed by Fisher.



Handy, easy-to-use Tissuemat® embedding compound offers choice of four precise melting points, won't crumble or crack in microtome, means perfectly prepared specimens. Tissuemat typifies array of reagents and specialties stocked by Fisher.

Glassware made sparkling clean — quickly, easily, economically — in new Fisher Standard Washer designed for small labs. It and Deluxe Washer are just two of the timesaving appliances Fisher carefully makes.

Want the full facts about how Fisher can help you? Details in free, data-packed bulletins. Clip, fill out and mail coupon to Fisher Scientific Company, 120 Fisher Building, Pittsburgh 19, Pa.

Fisher Scientific Company
120 Fisher Building
Pittsburgh 19, Pa.

Please send me the following information:

- ☐ "This Is Fisher"
- ☐ JEOL "Model JEM-6C" Electron Microscope
- ☐ Fisher "Tissuematon"
- ☐ Fisher Glassware Washers
- ☐ Fisher "Tissuemat"

Name_____ Title_____
Please Print

Company_____

Street_____

City_____ Zone_____ State_____

E-196

FISHER SCIENTIFIC

World's Largest Manufacturer-Distributor of Laboratory Appliances & Reagent Chemicals

Boston • Chicago • Fort Worth • Houston • New York • Odessa, Texas
Philadelphia • Pittsburgh • St. Louis • Washington • Montreal • Toronto

80% of Clinical Laboratories Agree...

Simplastin®

Here are some of the major reasons for SIMPLASTIN's use in 21,384,000 determinations of prothrombin time during 1960:

1. **Assured reproducible results.** 80% of clinical laboratories agree – by routine use – that SIMPLASTIN is the thromboplastin reagent th gives "best reproducibility"[1] of results.

2. **Lowered burden on laboratories.** For depen ability and ease in handling, laboratory directo select SIMPLASTIN, a quality controlled free dried thromboplastin extract. Already combin with calcium, it is prepared for use merely adding distilled water.

3. **Guaranteed controls** for pH, ionic streng suspended solids, particle size, moisture and s bility in varying temperatures.

4. **Contains no preservative** or artificial accel ators for prothrombin activation, as are found liquid preparations.

NOTE: In addition to routine laboratories, research laboratories are now using Simplastin in their basic coagulation studies. Research, too, is prone to the shortages of skilled assistance common to the routine laboratory. Maximum use of available skills is insured by getting away from reagent preparation in the thromboplastin area – where Simplastin's accuracy and reproducibility are difficult, indeed, to match. A bibliography of some reports of basic research, utilizing Simplastin and appearing in 1960 and 1961, appears on the bottom of this page. If you are investigating basic coagulation problems, write us for ways in which Simplastin can be of unique use in your problem areas. We will be happy to provide this Research Service without charge.

order Simplastin today, your safeguard of dependable results

GENERAL DIAGNOSTICS DIVISION **WARNER-CHILCOTT** DIV. MORRIS PLAINS

1. Carey, L. C. and Williams, R. D.: Ann. Surg. *152*:919, 1960. 2. Cronin, M. T. I. and Offenkrantz, F. M.: Am. J. Clin. Path. *34*:346, 19
3. Dinon, L. R. and Vander Veer, J. B.: Am. Heart J. *60*:6, 1960. 4. Kirk, J. E.: 14th Meeting: Council on Atherosclerosis of the Ameri
Heart Association, St. Louis. October, 1960. 5. Lewis, J. H.: J. Lab. & Clin. Med. *55*:245, 1960. 6. Matschiner, J. T. and Doisy, E. A.
Federation Proceeding 44th Annual Meeting, Chicago, Ill. April, 1960. 7. McGovern, J. J., Bunker, J. P., Goldstein, R. and Estes, J.
J.A.M.A. *175*:1011, 1961. 8. Messinger, W. J. and Weiner, M.: AMA Arch. Int. Med. *105*:727, 1960. 9. Perry, Seymour: AMA Arch.
Med. *105*:921, 1960. 10. Phillips, G. E., Luddecke, H. F., Breuchard, J. S. and Lenahan, J. G.: J. Lab. & Clin. Med. *56*:659, 1960. 11. Rub
stein, E. and Lack, A.: J. Appl. Physiol. *15*:598, 1960. 12. Smith, R., Rodman, T. and Pastor, B. H.: J.A.M.A. *174*:1917, 1960. 13. VieHi, T.
Murphy, T. P., James, J. A. and Pritchard, J. A.: J. Pediat. *56*:343, 1960. 14. Weiner, M. and Messinger, W. J.: J. Lab. & Clin. Med. *56*:7

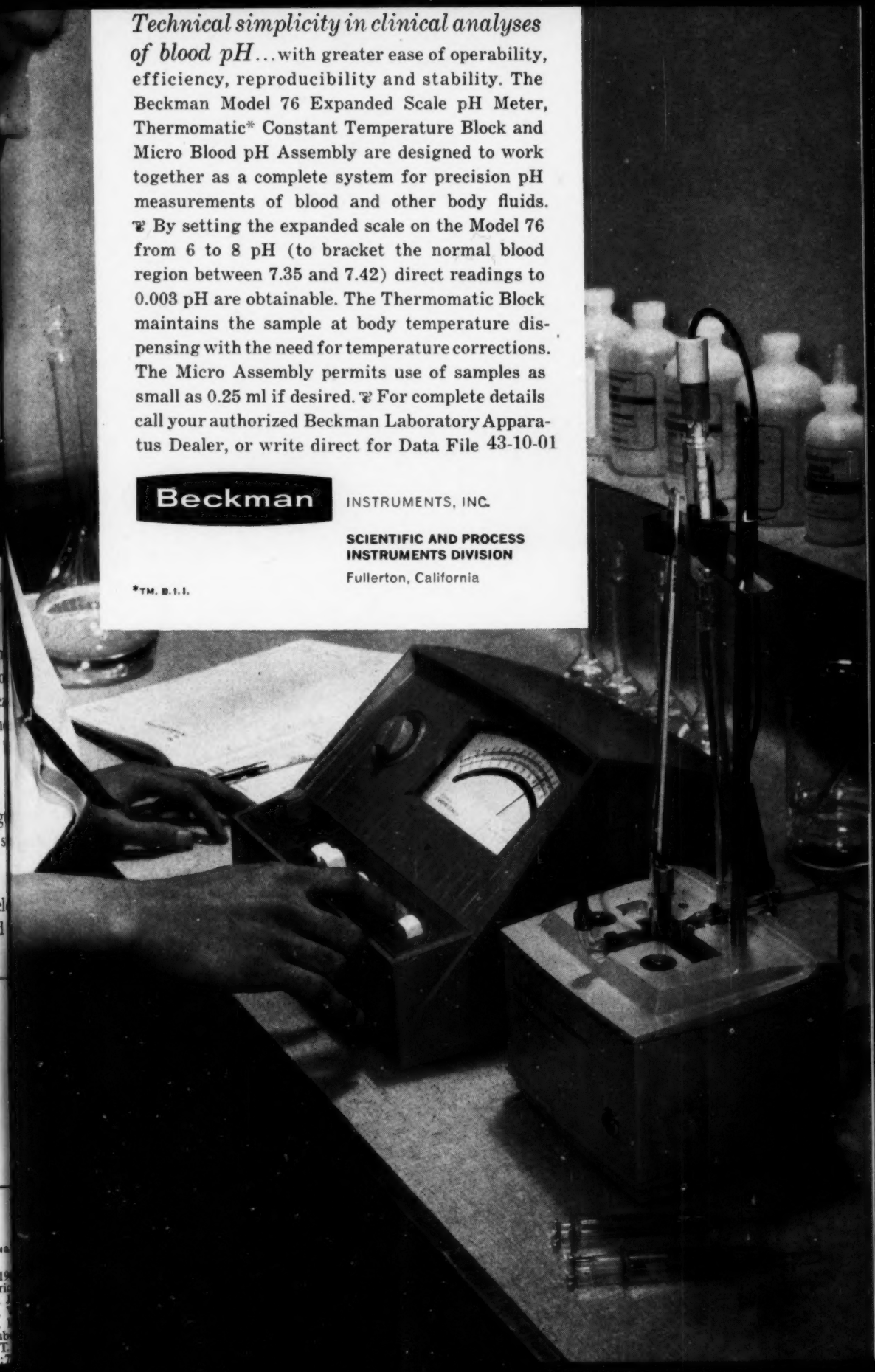
Technical simplicity in clinical analyses of blood pH... with greater ease of operability, efficiency, reproducibility and stability. The Beckman Model 76 Expanded Scale pH Meter, Thermomatic* Constant Temperature Block and Micro Blood pH Assembly are designed to work together as a complete system for precision pH measurements of blood and other body fluids. ❦ By setting the expanded scale on the Model 76 from 6 to 8 pH (to bracket the normal blood region between 7.35 and 7.42) direct readings to 0.003 pH are obtainable. The Thermomatic Block maintains the sample at body temperature dispensing with the need for temperature corrections. The Micro Assembly permits use of samples as small as 0.25 ml if desired. ❦ For complete details call your authorized Beckman Laboratory Apparatus Dealer, or write direct for Data File 43-10-01

Beckman® INSTRUMENTS, INC.

SCIENTIFIC AND PROCESS INSTRUMENTS DIVISION

Fullerton, California

*TM. B.I.I.



COLEMAN

Only the Coleman Junior adapts instantly to ultramicro spectrophotometry

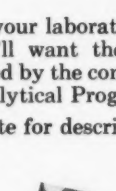
You can convert your Coleman Junior Spectrophotometer to microliter measurements in a matter of seconds.

Just insert the Coleman Ultramicro Cell Adapter in the instrument's cuvette well . . . that's all.

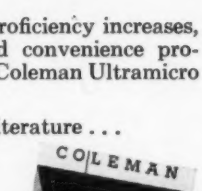
Adding the important capability of ultramicro analysis to your laboratory requires no massive expenditure for unproven, single-purpose equipment. The Coleman Program lets you get right into ultramicro analysis immediately on an extremely modest budget. Only $124.25 buys all the necessary adaptive equipment; another $44.50 provides you with all the necessary pipettes, volumetric ware and liquid containers for performing scaled-down versions of your own analytical procedures.

As your laboratory's proficiency increases, you'll want the added convenience provided by the complete Coleman Ultramicro Analytical Program.

Write for descriptive literature . . .



Bulletin EB-278 tells about ultramicro analysis on a budget.



Bulletin EB-263 describes the complete Coleman Ultramicro Analytical Program.

Order and simplification are the first steps toward mastery of any science

COLEMAN INSTRUMENTS, INC., MAYWOOD, ILLINOIS

ACCURACY... A TIMELESS RESPONSIBILITY

After hours" do not exist at Biochemical Procedures. The responsibility of deriving accurate, precise results from highly specialized clinical chemistry procedures cannot be limited by the traditional 8-hour working day. For this reason, Biochemical Procedures' laboratory departments remain staffed and operative 20-hours a day, 7 days a week.

Test processing begins immediately upon arrival of the specimen. Completion of the test procedure is unhampered by the time of day...or night. Results are air-mailed. Faster service and uncompromising accuracy...the reasons you should rely on Biochemical Procedures for specialized analyses.

Write for FREE MAILING CONTAINERS, complete FEE SCHEDULE and "THE BULLETIN OF LABORATORY MEDICINE," a monthly publication designed to keep you abreast of newest developments in the clinical laboratory field.

PROTEIN BOUND IODINE

The estimation of sub-microgram quantities of Protein Bound Iodine is a procedure fraught with technical problems. The procedure must be rigidly controlled using the finest techniques. Every day hundreds of P.B.I.'s are performed in duplicate at Biochemical Procedures, using both internal and external controls in all steps of the procedure. Our chemists are experts in the required technical manipulations necessary to attain accurate and precise results.

Serving hospitals, universities, laboratories, clinics and physicians throughout the world.

BIOCHEMICAL PROCEDURES, INC.

12012 Chandler Boulevard, North Hollywood, California, U.S.A.

BIOCHEMICAL PROCEDURES, INC.
12012 Chandler Boulevard, North Hollywood, California, U.S.A.

Please send the following material by return mail:

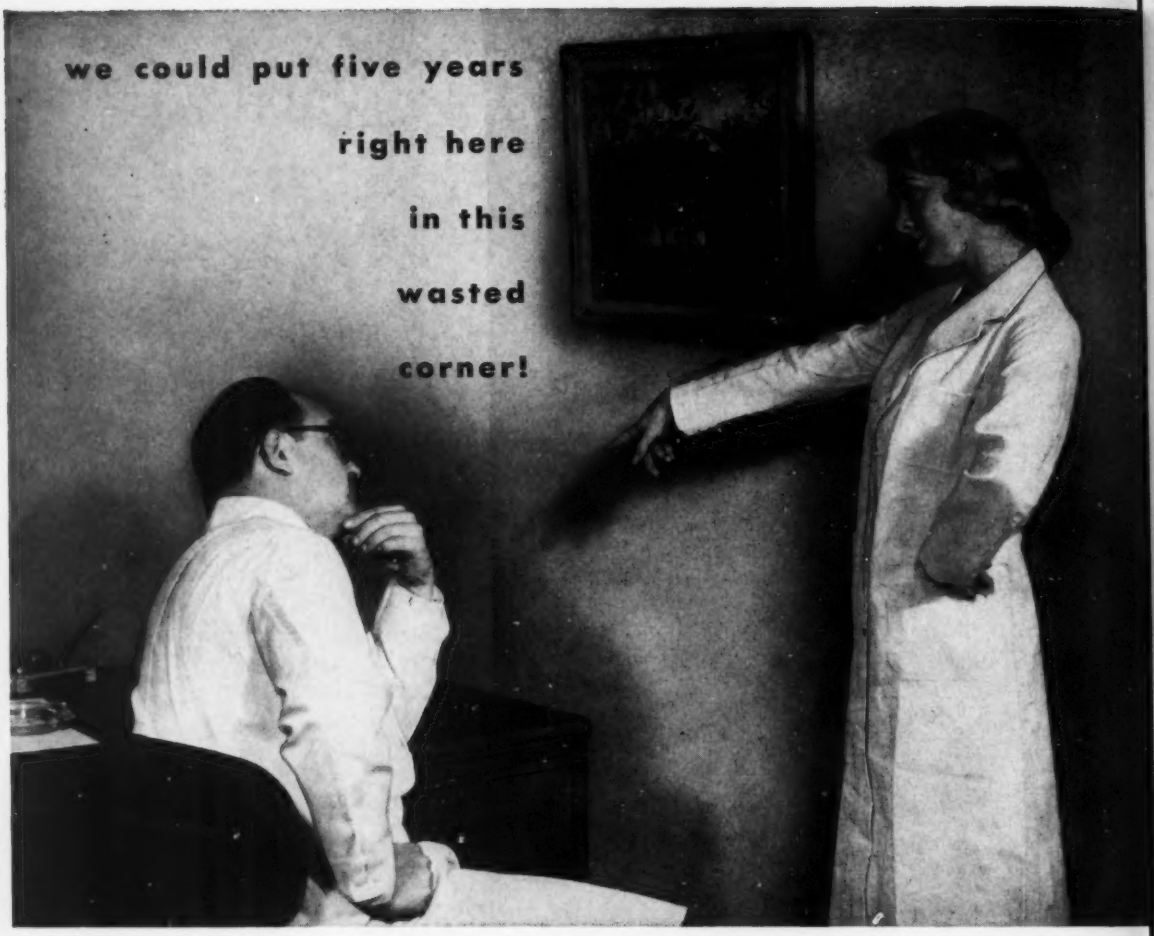
☐ **Mailing containers** ☐ **Fee schedule** ☐ **"The Bulletin of Laboratory Medicine"**

name _____

address _____

city _____ zone ____ state _____

JLCM 10-61



How many microslides do you average a day? Fifty, maybe?

A Lab-aid stack only 19" square and about five feet high would keep five years' output right at your fingertips.

That's because Lab-aid design gives you 45% more capacity, inch for inch, than conventional filing units. Think of it . . . a single unit section only five inches high, compact enough to fit handily on a desk top, will hold 6500 slides!

And that's not all . . . by interchanging different drawer sizes 1", 2", and 4", you can file Kodachromes, lantern slides, and index cards in the same cabinet. Other special-purpose cabinets (same basic dimensions so they all stack together) let you file fresh slides flat in spread-out trays, or file paraffin blocks in shallow drawers.

The beauty of it all is that you get all these advantages at no greater filing cost-per-slide than ordinary cabinets.

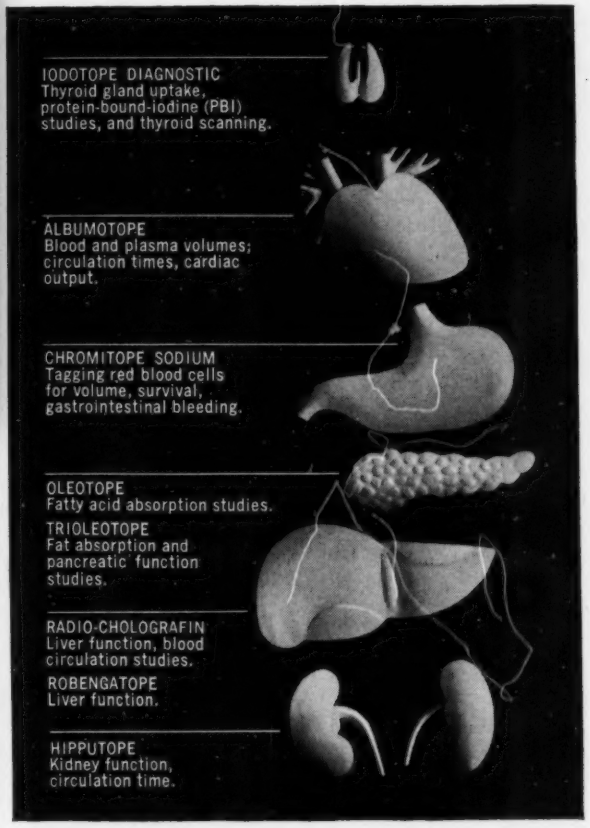
TECHNICON Lab-aid®

laboratory filing system

Bulletin No. 14L-56 tells the story. Let us send it to you.

THE TECHNICON COMPANY, CHAUNCEY, N. Y.

Medotopes by Squibb—
Radioactive Pharmaceuticals for diagnosis and therapy



Medotopes cover an extensive range of diagnostic and therapeutic procedures, each reflecting the latest developments in radioactive medicine. *All* Medotopes convey, as well, the utmost in safety and convenience. *All* feature unique packaging safeguards: exclusive lead shield enclosures for easy opening—no direct contact is required; bottle caps unscrew automatically; shipping cartons have "pull-tab" openers; vials and bottles are carefully encased and then doubly protected by transparent, shatterproof plastic coatings. Each preparation is custom-handled. Delivery is custom-routed by Squibb Traffic Service. Access to three major airports expedites this service.

***Diagnostic Medotopes*, in addition to those illustrated, are: Chromitope Chloride • Ferrutope • Phosphotope • Rubratope 58 and 60 • Cobatope 58 and 60 Tritiotope • *Therapeutic Medotopes* are: Aureotope • Chromphosphotope • Iodotope • Iriditope • Phosphotope.**

For full information, communicate with E. R. Squibb & Sons, Professional Service Department, 745 Fifth Avenue, New York 22, New York.

MEDOTOPES SQUIBB

Medotopes

SQUIBB
Squibb Quality
—the Priceless Ingredient

'ALBUMOTOPE'®, 'AUREOTOPE'®, 'CHOLOGRAFIN'®, 'CHROMITOPE'®, 'CHROMPHOSPHOTOPE'®, 'COBATOPE 58 AND 60'®, 'FERRUTOPE'®, 'HIPPUTOPE', 'IODOTOPE'®, 'IRIDITOPE'®, 'MEDOTOPES'®, 'OLEOTOPE'®, 'PHOSPHOTOPE'®, 'ROBENGATOPE'®, 'RUBRATOPE'®, 'TRITIOTOPE'®, AND 'TRIOLEOTOPE'® ARE SQUIBB TRADEMARKS.

Need Laboratory Supplies or Equipment?

FOR *Instant Service* CONTACT

HARSHAW

PYREX · COORS · KIMAX · BARNSTEAD | BECKMAN · COLEMAN · INT. EQ. CO. · LECO | AINSWORTH · BECKER · SARTORIUS · OHAUS | B & L OPTICAL · AMER. OPTICAL · ZEISS • LEITZ · BUEHLER | E & D · WHATMAN FILTER PAPER · S & S | MC & B · J. T. BAKER · MALLINCKRODT · GLAS-COL | PRECISION SC. CO. · LAB. FURN. CO. · LABLINE · BLUE M

Cleveland | Cincinnati | Detroit | Houston | Los Angeles | Oakland | Philadelphia

We invite your consideration of the following items:

DYNAZOOM® MICROSCOPES

Zoom up, zoom down at the twist of a dial with the new Bausch & Lomb DynaZoom® Laboratory Microscopes. The revolutionary MicroZoom® optical system makes "step magnification" obsolete. You can study and photograph specimens at any power from 17.5X to 1940X!

Price range: same as before. Flexible: choice of six microscope bodies which are interchangeable on basic stand. Ask us for Bulletin D-185 and price list.

Each . . . . . Prices start at **$229.00**

LABORATORY CARTS

Save time, cut down on breakage and move things the sensible way—on lab carts (all stainless steel).

H-7915 Heavy Duty Cart (illustrated). Carrying capacity is 400 pounds. Overall: 17¾″ x 30″ x 32½″ high. With guard rails.

Each **$67.00**

H-7916 Utility Cart. Carrying capacity is 200 pounds. Overall: 15½″ x 27″ x 31½″ high. Without guard rails.

Each **$35.75**

(Note: on West Coast $71.00 and $37.95 respectively)

HARSHAW SCIENTIFIC

Division of The Harshaw Chemical Co. • Cleveland 6, Ohio

SUPPLYING THE NATION'S LABORATORIES FROM COAST TO COAST

HARSHAW

SALES BRANCHES AND WAREHOUSES

CINCINNATI 13, OHIO — 6265 Wiehe Road
HOUSTON 11, TEXAS — 6622 Supply Row
OAKLAND 1, CAL. — 5321 East 8th Street
CLEVELAND 6, OHIO — 1945 East 97th Street
DETROIT 28, MICH. — 9240 Hubbell Ave.
LOS ANGELES 32, CAL. — 3237 So. Garfield Ave.
PHILADELPHIA 48, PA. — Jackson & Swanson Sts.

SALES OFFICES • Baton Rouge 6, La. • Buffalo 2, N. Y. • Hastings-On-Hudson 6, N. Y. • Pittsburgh 22, Pa.

Introducing HANDI-BOY* Jr. 2 gallon unit.

...same convenience and advantages of the HANDI-BOY* (5 gallon).

*Trademark, Hartman-Leddon Company, Inc.

Approximately 2,000 different Harleco reagents are available in these unique dispensing units; write for details.

Harleco®

HARTMAN-LEDDON COMPANY

60th & Woodland Avenue • Philadelphia 43, Pennsylvania

is pharmaceutical advertising really "advertising"?

of course it is, though some have called it "education" . . . not <u>really</u> "advertising."

Of course it's "advertising"... a frankly competitive activity of the American private enterprise system to which this industry belongs. Of course it's "advertising"...created in the hope of getting the physician to note and read; of persuading him, by setting forth proven indications and advantages, to learn about a drug; and of thereby helping him alleviate suffering or cure disease by prescribing it.

"Advertising"? Surely! BUT indisputably different from any other advertising in the world (which is just what has led people to devise various different names for it). For in its proper role it communicates the vital information . . . good, bad, and indifferent . . . and it keeps the physician abreast of each useful new clinical application and each new danger revealed during increasing use of the drug.

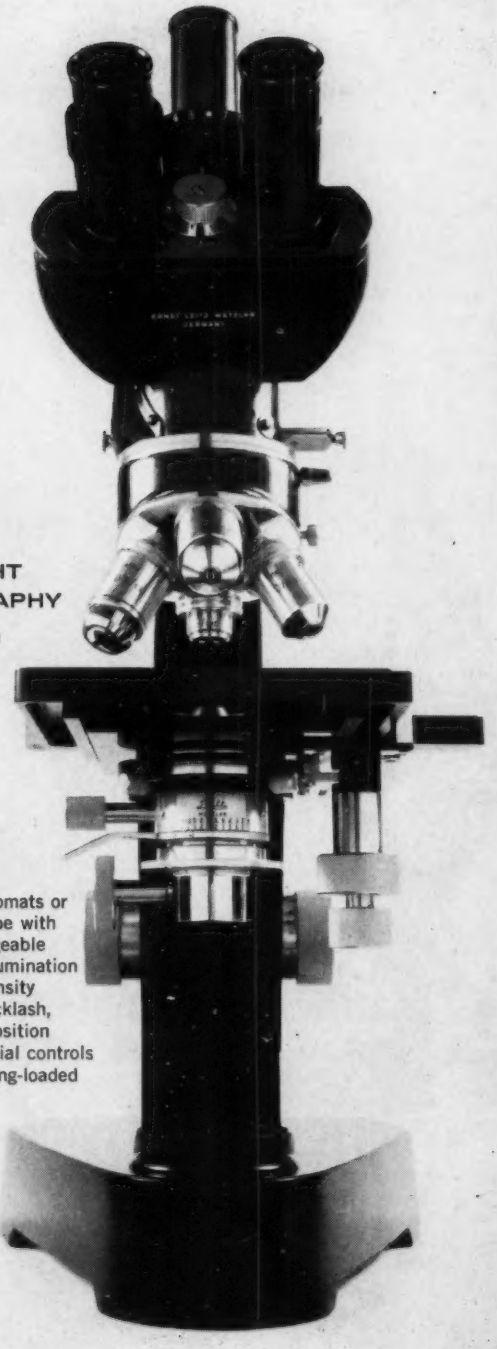
There's been a lot of talk about "over-advertising", and there may have been occasional excesses. But consider the potential dangers, in this era of astonishing new drugs, of "under-advertising". . . in view of the complexity of modern drug therapy; the lag of 6 to more than 18 months before the appearance of definitive medical articles on new drugs; and the fact that there is no other source of such comprehensive information about a new agent as the company that ran it through the crucial gauntlet of animal pharmacology and clinical investigation.

This message is brought to you on behalf of the producers of prescription drugs. For additional information, please write Pharmaceutical Manufacturers Association, 1411 K Street, N.W., Washington 5, D.C.

LEITZ LABOLUX

specialized versatility for

6 ways of seeing



THE LABORATORY MICROSCOPE THAT FULFILLS RESEARCH REQUIREMENTS

1 BRIGHT FIELD **2** DARK FIELD
3 PHASE CONTRAST **4** INCIDENT LIGHT
5 FLUORESCENCE **6** PHOTOMICROGRAPHY

The technical highlights below are your assurance that Leitz has given its traditional exacting attention to the perfection of each individual function of the Labolux, despite its almost infinite variety of interchangeable combinations. It's the only truly **versatile** microscope that can be **specialized** to meet the critical standards of experts in each field of application.

TECHNICAL HIGHLIGHTS

* Magnification from 45 to 1250X * Achromats, Apochromats or the new Plano-Objectives * Inclined 1.25X binocular tube with interpupillary adjustment * 360° rotation of interchangeable instant-lock Binocular and Monocular tubes * Built-in illumination with appropriate transformer provides variable light intensity * Interchangeable condensers * Exclusive Leitz anti-backlash, single-knob coarse and fine focusing * Convenient low-position controls * Built-in graduated mechanical stage with coaxial controls * Quadruple interchangeable revolving nosepiece * Spring-loaded medium and high-power objective mounts * FS tube for binocular observation and photomicrography * Easily adapted for use with the new ORTHOMAT

Leitz

E. LEITZ, INC., 468 PARK AVENUE SOUTH, NEW YORK 16, N. Y.
Distributors of the world-famous products of Ernst Leitz G.m.b.H., Wetzlar, Germany—Ernst Leitz Canada Ltd.
LEICA AND LEICINA CAMERAS • LENSES • PROJECTORS • MICROSCOPES

E. LEITZ, INC., 468 Park Avenue South, New York 16, N.Y.

Gentlemen:

☐ Please send me complete information on the LABOLUX.

☐ Kindly have Leitz representative phone for appointment to demonstrate the LABOLUX at no obligation to me.

Name _____

Address _____

City _____ Zone ____ State ____

Telephone _____

41061

Paragon Reagents Paramount Quality

Tested and proven reagents of the very highest quality

Kaiserling Powder No. 1—PARAGON
Kaiserling Powder No. 3—PARAGON

For preparing Kaiserling solutions No. 1 and No. 3 used in the fixation and color preservation of anatomical and pathological museum specimens. Perfect fixation and color preservation are now readily obtained. Either solution may be easily prepared at once by dissolving the pre-weighed powder in hot water and allowing to cool. The small packages of powder conserve space and solve storage problems for these solutions. Large amounts of liquid formalin need no longer be kept on hand. Gallons may be prepared in a matter of minutes. Full directions appear on label.

PS1661	*Kaiserling Powder No. 1—Paragon*	*For a gallon*	*$1.85*
PS1651	*Kaiserling Powder No. 1—Paragon*	*For a liter*	*1.25*
PS1663	*Kaiserling Powder No. 3—Paragon*	*For a gallon*	*1.85*
PS1653	*Kaiserling Powder No. 3—Paragon*	*For a liter*	*1.25*

Zenker's Fluid—PARAGON

An excellent general fixative which is specially recommended for the fixation of blood forming tissues and organs. Supplied without acetic acid which is added just prior to use. Zenker formol (Helly's fluid) may be prepared by adding formalin. Full instructions on bottle label. The best fixative for obtaining the sharpest nuclear details with hematoxylin stains.

PS1674	*Zenker's Fluid—Paragon*	*Bottle (500 cc)*	*3.25*

Bouin's Fluid—PARAGON

Specially recommended for the fixation of skin tumors. It penetrates with a minimum of hardening and shrinkage and will not overfix. It is noteworthy for the production of brilliant red counterstains with eosin.

PS1675	*Bouin's Fluid—Paragon*	*Bottle (500 cc)*	*2.25*

Schaudinn's Fluid—PARAGON

For use in parasitology. Fixes and preserves cellular details of ova and parasites. Excellent for fecal smears. Preserves parasites without shrinkage or distortion so that identification is easier. Supplied without acetic acid which is added before use.

PS1673	*Schaudinn's Fluid—Paragon*	*Bottle (500 cc)*	*3.25*
PS1672	*Schaudinn's Fluid—Paragon*	*Bottle (250 cc)*	*2.00*

Write for catalogs 957A & 957B for more detailed information and staining technics

All prices F. O. B. New York, New York, subject to change without notice

Manufactured exclusively by

PARAGON C. & C. CO., INC., 2540 Belmont Ave., New York 58, New York

Cable address: Wijeno, New York

Paragon Reagents • Dependable • Tested • Paramount Quality

Paragon Standards and Reagents for Clinical Chemistry

Accurately prepared and standardized in our own laboratories

Description	Catalog No.	Quantity	Price
Acid Digestion Mixture—Paragon (concentrated)	P745	100 cc	$1.20
Alkaline Copper Tartrate—Paragon (concentrated)	P851	for 1 liter	.45
Ammonium Sulfate Solution—Paragon (saturated-aqueous)	P582	100 cc	1.00
Benedict Compound Paragon—Qualitative, granules	P520	for 1 gallon	1.35
	P501	for 1 liter	.45
Benedict Compound Paragon—Quantitative, granules	P631	for 1 gallon	2.70
	P611	for 1 liter	.90
Paragon Bone Decalcifying Solution	PS3100	500 cc	2.35
Benzoic Acid Solution—Paragon (0.25% aqueous)	P890	500 cc	1.00
Paragon Combined Decolorizer-Counterstain	PS2275	500 cc	1.80
Creatinine Standard—Paragon (stock—1 ml. equals 1 mg.)	P360	100 cc	.75
Creatinine Standard—Paragon (working—1 ml. equals .006 mg.)	P370	100 cc	.75
Diazo Reagent A—Paragon (0.1% sulfanilic acid)	P575	250 cc	.80
Diazo Reagent B—Paragon (0.5% sodium nitrate)	P576	100 cc	.75
Ferric Ammonium Sulfate—Paragon (ferric alum powdered)	P676	50 gm.	1.45
Ferric Chloride Solution—Paragon (10% aqueous)	P970	250 cc	.90
Glucose Standard—Paragon (stock—1%)	P870	100 cc	1.00
Glucose Standard—Paragon (intermediate—stock—0.2%)	P872	100 cc	1.00
Glucose Standard—Paragon (1 ml. equals 0.1 mg.)	P875	100 cc	1.00
Glucose Standard—Paragon (1 ml. equals 0.2 mg.)	P876	100 cc	1.00
Icteric Index Standard—Paragon (0.01% potassium bichromate)	P590	250 cc	.85
Nessler Compound Paragon, granules	P701	for 1 liter	1.90
Nitrogen Standard—Paragon (stock—1 ml. equals 1 mg.)	P722	100 cc	.85
Nitrogen Standard—Paragon (working—1 ml. equals 0.1 mg.)	P723	100 cc	.85
Nitrogen Standard—Paragon (working—1 ml. equals 0.05 mg.)	P724	100 cc	.85
Nitrogen Standard—Paragon (stock—1 ml. equals 0.2 mg.)	P725	100 cc	.85
Nitrogen Standard—Paragon (stock—1 ml. equals 1.5 mg. in 0.1N sulfuric acid)	P726	100 cc	1.00
Nitrogen Standard—Paragon (working—1 ml. equals 0.015 mg. in 0.01N sulfuric acid)	P721	100 cc	1.00
Nitrogen Standard—Paragon (stock—1 ml. equals 0.75 mg.)	P728	100 cc	.85
Nitrogen Standard—Paragon (working 1 ml. equals 0.015 mg.)	P729	100 cc	1.00
Phenophthalein Solution—Paragon (1% alcoholic)	P960	100 cc	.85
Phospho-Molybdic Acid Reagent—Paragon, concentrated	P801	for 1 liter	2.35
Picric Acid Solution—Paragon (especially purified—saturated—aqueous)	P350	500 cc	1.50
Standard Potassium Thiocyanate Solution—Paragon (1 ml. equals 1 mg. chlorine)	P651	250 cc	1.00
Standard Potassium Thiocyanate Solution—Paragon (1 ml. equals 1 mg. sodium chloride)	P656	250 cc	1.00
Standard Silver Nitrate Solution—Paragon (1 ml. equals 1 mg. chlorine)	P650	250 cc	.75
Standard Silver Nitrate Solution—Paragon (1 ml. equals 1 mg. sodium chloride)	P655	250 cc	.75
Sodium Alizarin Sulfonate Solution—Paragon (1% aqueous)	P965	100 cc	.60
Sodium Hydroxide Solution—Paragon (10%)	P355	500 cc	1.50
Sodium Hydroxide Solution—Paragon (0.1N)	P950	500 cc	1.20
Sodium Tungstate Solution—Paragon (10%)	P116	500 cc	2.25
Sulfuric Acid—Paragon (2/3N)	P120	500 cc	1.20
Sulfuric Acid—Paragon (1/12N)	P125	500 cc	1.20
Toepfer's Reagent—Paragon (0.5% alcoholic)	P955	100 cc	1.00
Van den Bergh Standard—Paragon (cobalt sulfate)	P580	250 cc	1.50

Write for catalogs 957A & 957B fully describing Paragon Staining Solutions, Chemical Reagents and Solutions and Paragon Tray Drawer Cabinets and accessories

All prices F.O.B. New York, N. Y., subject to change without notice.

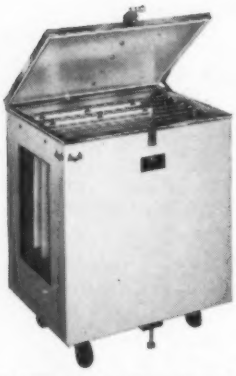
Manufactured exclusively by

PARAGON C. & C. CO., Inc., 2540 Belmont Ave., New York 58, N. Y.

Cable Address: Wijeno, New York

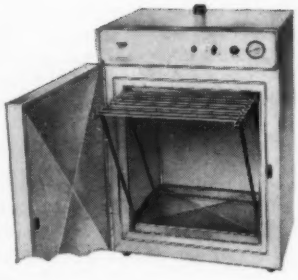
Thomas selection for paper, column and gas

CHROMATOGRAPHY
and ELECTROPHORESIS

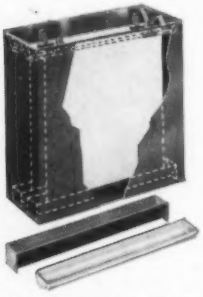


3102-B06. Thomas Chromatography Cabinet, Stainless steel lined, with four solvent assemblies for up to eight 18¼ x 22½ in. paper chromatograms by descending or ascending techniques; 34 inches high . . **470.00**

3108-B05. Thomas-Kolb Chromatography Jars. Sturdy economical glass jars for ascending technique. Small takes papers up to 6-in. square; Large up to 10-in. square.
Small . . . . . **22.90** Large **25.40**



3127-C10. National Chromatographic Oven, Stainless steel chamber with swing-out rack for easy loading. Range, ambient to 120°C. Takes ten papers up to 18¼ x 22½ in. For 115 volts, a.c. **875.00**



3106-F05. Thomas-Mitchell Chromatography Tank, Stainless steel. A widely used, compact, economical tank for two 8-in. square paper chromatograms by ascending technique. With two Stainless steel troughs **29.85**
NOTE—Accessories include adapters for descending and for continuous ascending techniques.

Column Chromatography

Pyrex® and Kimax® glass Chromatographic Tubes

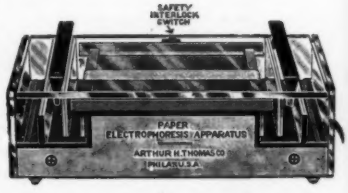
Fischer & Porter Chromatographic Columns

Whatman Cellulose Powder and Ion Exchangers

Dowex® Ion Exchange Resins

Chromosorb®

Electrophoresis Apparatus



4937-W5. Thomas Paper Strip Electrophoresis Apparatus. Platinum foil electrodes; 10¼-in. migration chamber in transparent methacrylate cabinet with double-pole safety interlock. With variable Power Supply. For 115 volts, 50 or 60 cyc., a.c. . . . **344.50**

The 1961 edition of our general catalog lists more than 300 items specially developed for Paper, Column and Gas Chromatography and for Paper Electrophoresis. Some of the specialties developed by us are shown above. In addition, we list and stock:

Thomas Glass Chromatography Jar, for six chromatograms 9⅛ x 22½ inches

"Precision" Centrifugal High Speed Chromatograph

Rinco Automatic Fraction Collectors (two models)

Chromato-Vue Ultraviolet Lamp

Beckman Gas Chromatographs, Models GC-1 and GC-2

"Precision" Chronofrac, Model VP-1

Spinco Model R Paper Strip Electrophoresis System

Photovolt Densitometers, Models 525, 53 and 542

For detailed information, see following sections of our new 1961 catalog: pp 228-241 and 432-440. Separate leaflets mailed upon request.

QUALITY AND SERVICE
A.H.T.CO.
PHILA. U.S.A.
LABORATORY APPARATUS

ARTHUR H. THOMAS COMPANY

More and more laboratories rely on Thomas / Laboratory Apparatus and Reagents

VINE ST. AT 3RD • PHILADELPHIA 5, PA.

The Journal of
LABORATORY and CLINICAL MEDICINE

Volume 58 Number 4 OCTOBER 1961

Clinical and experimental

Effects of steroid-17-spirolactones on aldosterone secretion, excretion, and metabolism in man

EUGENE T. DAVIDSON, M.D., WILLIAM S. COPPAGE, JR., M.D., DONALD ISLAND, B.S., and GRANT W. LIDDLE, M.D. *Nashville, Tenn.*

The steroid-17-spirolactones induce natriuresis by an action which is antagonistic to that of aldosterone. They reverse all of the actions of aldosterone on electrolyte excretion, and are ineffectual in the absence of aldosterone or similar corticosteroids. They have no apparent direct effect upon aldosterone secretion, metabolism, or excretion. Spirolactone-induced natriuresis may lead to increased aldosterone output. Urinary 17-hydroxycorticosteroids and 17-ketosteroids are not appreciably affected by spirolactones in courses of treatment up to 1 week in duration. The present study supports the concept that the spirolactones modify electrolyte excretion through peripheral antagonism of the action of aldosterone.

The observation by Landau and his co-workers, in 1955,[1] that progesterone blocks the sodium-retaining action of desoxycorticosterone, served as a lead to the development by Cella and Kagawa[2, 3] and Van Arman[3] of a series of steroid-17-spirolactones which promote sodium excretion in rats, dogs, and man.[4, 5] The concept has been advanced that "spirolactones" are peripheral antagonists of aldosterone because they reverse all the effects of aldosterone on electrolyte excretion but have little or no effect in the absence of aldosterone or other sodium-retaining steroids. The purpose of the study

From the Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tenn.

Supported by Grants Nos. A-1748 and 2A-5092 from the United States Public Health Service.

Received for publication Dec. 16, 1960.

presented in this paper was to determine whether, in addition to the peripheral antagonism of aldosterone, the spirolactones may also influence the adrenal secretion, peripheral metabolism, or renal excretion of aldosterone.

Singer[6] found that, when administered to rats maintained on a diet high in sodium, a spirolactone induced only slight increases (not statistically significant) in aldosterone secretion rate. However, when administered to rats on restricted sodium intake, the spirolactone induced distinct increases in aldosterone secretion. Singer inferred that spirolactone had no direct effect on adrenocortical secretion of aldosterone but that, by inducing a greater degree of sodium depletion in the rats on restricted intake, it was capable of bringing about indirect stimulation of aldosterone secretion.

Slater and associates[7] observed inconsistent changes in aldosterone secretion and excretion while using a spirolactone in the treatment of edematous patients.

Materials and methods

Eight subjects participated in this study. Five were normal volunteers, one had primary aldosteronism, one nephrosis, and one cirrhosis with ascites. Each subject received a chemically constant diet throughout the study. All medications were constant throughout any one study except for spirolactones, which were administered intramuscularly or orally over periods of 3 to 6 days, and tritium-labeled d, l-aldosterone, which was administered intravenously in dosage of 50 μg (20 μc) for the purpose of determining aldosterone secretion rates in 2 of the studies. Daily 24 hour collections of urine

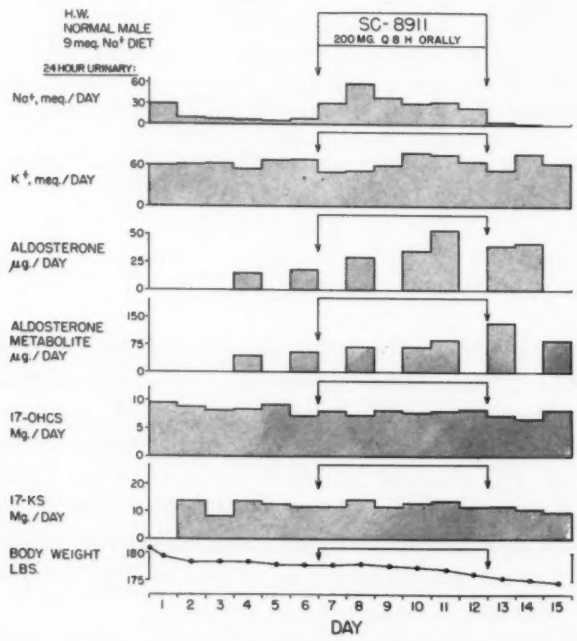


Fig. 1. Urinary Na^+, K^+, aldosterone, and aldosterone metabolite (pregnane-3, 18, 21-triol-11, 20-dione) excretion before, during, and after SC-8,911 administration to a normal man on a constant low sodium diet.

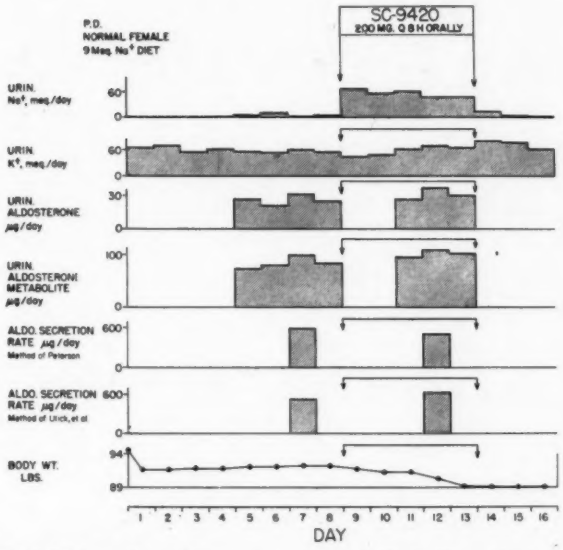


Fig. 2. Effects of SC-9,420 on the urinary excretion of Na^+, K^+, aldosterone, and aldosterone metabolite and on the adrenal secretion of aldosterone in a normal woman on a low sodium diet.

were made, using refrigeration and thymol to inhibit bacterial activity. Aldosterone was extracted from the urine at pH 1.0 with dichloromethane and was quantitated by the isotope derivative technique of Kliman and Peterson.[8] The urinary "tetrahydro" metabolite of aldosterone (pregnane-3, 18, 21-triol-11, 20-dione) was measured by a modification of the method of Ulick and colleagues.[9] Aldosterone secretion rates were determined from the specific activity of both urinary aldosterone and urinary pregnane-3, 18, 21-triol-11, 20-dione during the 24 hour period following the injection of a tracer dose of tritium-labeled aldosterone. Sodium and potassium were measured by flame photometry.

17-Hydroxycorticosteroids were determined by a modification[10] of the method of Silber and Porter.[11] When subjects were being treated with the spirolactone SC-9,420 it was found that chromogenic artifacts appeared in the urine, which interfered with the determination and necessitated the introduction of the following steps: (1) A preliminary extraction of the urine was performed by shaking once with 8 volumes of carbon tetrachloride, once with 8 volumes of isooctane, and twice with 1 volume of benzene. The benzene extracts were then back extracted with 2 volumes of water. This treatment extracted most of the SC-9,420 artifacts into the organic solvents without removing significant amounts of the true 17-hydroxycorticosteroids such as cortisol, tetrahydrocortisone, and tetrahydrocortisol. Urine samples collected during control periods were subjected to the same preliminary extractions as were found necessary for those collected during treatment with SC-9,420. (2) As a further precaution an absorption-curve correction was employed in the final calculation of Porter-Silber chromogen in order to correct for any remaining SC-9,420 contaminants, which absorb maximally at 450 mμ. (3) In selected cases the identities and quantities of cortisol, cortisone, tetrahydrocortisol, and tetrahydrocortisone were verified with the aid of multiple system paper chromatography.

17-Ketosteroids were measured by the Zimmermann reaction as described by Callow and associates,[12] using urinary extracts prepared by the method of Drekter and coworkers,[13] and employing the color correction equation suggested by Allen.[14] During spirolactone administration the Zimmermann chromogens were extracted into dichloromethane just prior to reading optical densities.

Table I. Effects of spirolactones upon excretion of Na^+, K^+, and steroids and upon aldosterone secretion rate

Subject, diagnosis, and dietary Na^+	*Day of study*	*Spirolactone*	*Urinary aldosterone (μg/day)*	*Urinary 17-hydroxycorticoids (mg./day)*	*Urinary 17-ketosteroids (mg./day)*	*Urinary Na^+ (mEq./day)*	*Urinary K^+ (mEq./day)*
M. S. (Normal	1	0	17	7	10	9	69
woman, 9 mEq.	2	0	—	9	10	10	78
Na^+)	3	0	21	10	11	5	81
	4	0	—	10	11	7	88
	5	0	18	9	11	4	77
	6	0	—	10	12	5	84
	7	SC-9,420*; 200 mg. orally every 8 hours	24	9	12	56	67
	8	SC-9,420; 200 mg. orally every 8 hours	—	10	11	101	76
	9	SC-9,420; 200 mg. orally every 8 hours	42	10	11	78	68
	10	SC-9,420; 200 mg. orally every 8 hours	—	9	10	43	88
	11	SC-9,420; 200 mg. orally every 8 hours	54	10	9	31	91
	12	SC-9,420; 200 mg. orally every 8 hours	—	9	9	27	87
	13	0	120	10	10	13	98
	14	0	—	10	9	1	67
	15	0	67	10	10	0	84
L. T. (Cirrhosis,	1	0	—	6	4	51	67
22 mEq. Na^+)	2	0	—	6	3	34	55
	3	0	—	6	3	56	52
	4	0	8	6	4	57	47
	5	SC-9,420; 200 mg. orally every 12 hours	7	5	4	108	32

	6	SC-9,420; 200 mg. orally every 12 hours	13	—	—	131	43
	7	SC-9,420; 200 mg. orally every 12 hours	15	—	—	129	69
	8	SC-9,420; 200 mg. orally every 12 hours	12	7	5	183	74
	9	SC-9,420; 200 mg. orally every 12 hours	23	6	5	129	63
	10	SC-9,420; 200 mg. orally every 12 hours	30	5	5	103	54
	11	0	28	6	5	113	66
	12	0	26	6	5	106	80
	13	0	28	7	5	58	88
W. N. (Normal man, 19 mEq. Na^+)	1	0	22	9	12	10	66
	2	0	—	10	11	4	52
	3	0	26	11	12	4	63
	4	0	—	9	12	6	63
	5	0	20	10	9	8	57
	6	SC-8,109†; 250 mg. orally every 8 hours	24	7	11	78	38
	7	SC-8,109; 250 mg. orally every 8 hours	—	7	9	108	44
	8	SC-8,109; 250 mg. orally every 8 hours	30	6	12	86	60
	9	SC-8,109; 250 mg. orally every 8 hours	—	7	8	71	62
	10	SC-8,109; 250 mg. orally every 8 hours	—	8	6	54	79

Table I—Cont'd

Subject, diagnosis, and dietary Na^+	*Day of study*	*Spirolactone*	*Urinary aldosterone (μg/day)*	*Urinary 17-hydroxycorticoids (mg./day)*	*Urinary 17-ketosteroids (mg./day)*	*Urinary Na^+ (mEq./day)*	*Urinary K^+ (mEq./day)*
	11	SC-8,109; 250 mg. orally every 8 hours	80	9	7	45	73
	12	0	—	11	6	13	87
	13	0	100	10	6	3	83
	14	0	—	8	7	2	75
	15	0	—	10	9	1	68
	16	0	96	11	7	1	62
				Urinary "tetrahydroaldosterone" (μg/day)	*Aldosterone secretion rate (μg/day)*		
J. J. (Normal man, 9 mEq. Na^+)	1	0				2	115
	2	0	45	219	—	3	111
	3	0	54	196	—	1	112
	4	0	59	225	1,350	4	116
	5	0	47	180	—	3	123
	6	SC-9,420*; 200 mg. orally every 8 hours	—	—	—	19	115
	7	SC-9,420; 200 mg. orally every 8 hours	—	—	—	17	124
	8	SC-9,420; 200 mg. orally every 8 hours	45	207	—	25	123
	9	SC-9,420; 200 mg. orally every 8 hours	50	187	1,170	35	137
	10	SC-9,420; 200 mg. orally every 8 hours	46	185	—	37	109

*SC-9,420 is the 7α acetylthio derivative of SC-5,233 (3-[3-oxo-17β-hydroxy-4-androsten-17α-yl] propionic acid γ-lactone).
†SC-8,109 is the 19-nor of SC-5,233.

The spirolactones employed in this study are designated by code numbers for the sake of convenience. "SC-5,233" is 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone. The other compounds are derivatives of SC-5,233. "SC-8,911" is the 1-2 dehydro, "SC-8,109" is the 19-nor, and "SC-9,420" is the 7α-acetylthio derivative of SC-5,233. The official pharmaceutical name of SC-9,420 is "spironolactone." Qualitatively, all of these agents were similar in their effects on urinary electrolyte excretion. Quantitatively, all 3 derivatives were found to be more potent than the original compound SC-5,233.

Results

Effects of spirolactones on urinary electrolytes. As in previous studies, the spirolactones induced increases in urinary sodium and chloride and decreases in urinary potassium, ammonium, and titratable acid in subjects with intact adrenal glands.

Effect of spirolactones on urinary aldosterone levels. There was either no significant change or an increase in aldosterone excretion during and following administration of spirolactones. In no case was a diminution of aldosterone excretion observed as a result of spirolactone therapy (Figs. 1 through 3 and Table I).

Effect of spirolactones on urinary "tetrahydroaldosterone." Urinary levels of the tetrahydro metabolite of aldosterone were either unchanged or increased during and following administration of spirolactones. The pattern of excretion of this metabolite before and during spirolactone administration resembled that of aldosterone. In no instance was a decrease in aldosterone metabolite excretion observed as a result of spirolactone therapy (Figs. 1 and 2 and Table I).

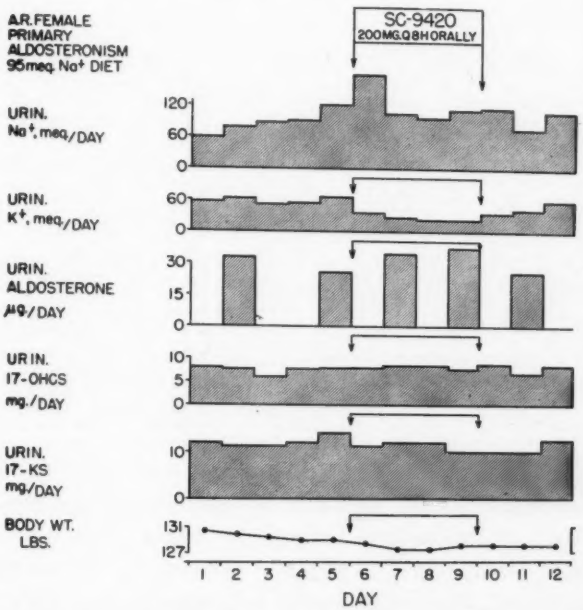


Fig. 3. Effect of SC-9,420 upon electrolyte and steroid excretion in a patient with primary aldosteronism.

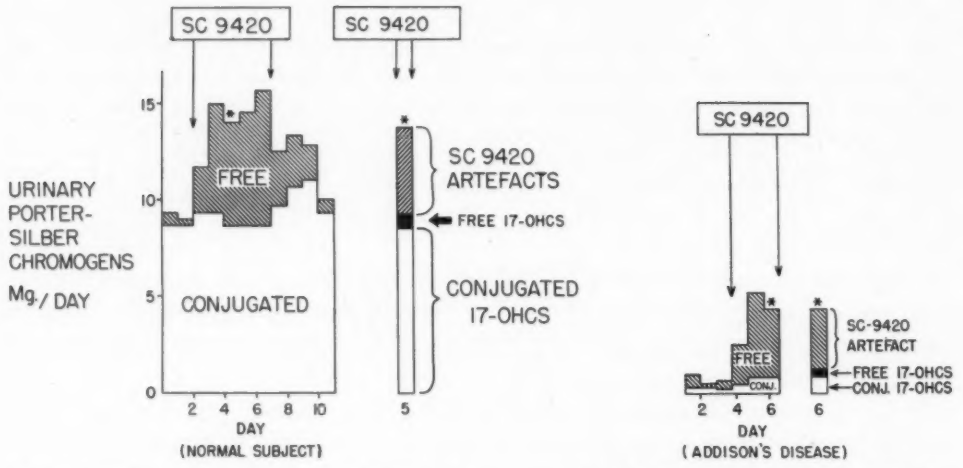


Fig. 4. Analyses of urinary Porter-Silber chromogens before, during, and after administration of SC-9,420 to a normal subject (left) and to a patient with Addison's disease (right). In each case SC-9,420 caused a substantial increase in the "free" fraction (material extractable from urine with dichloromethane without prior hydrolysis with β-glucuronidase). Porter-Silber chromogen glucuronides ("conjugates") were unaffected by SC-9,420. Specimens indicated by the asterisks (*) were further fractionated by means of various solvents and by paper chromatography. Results are indicated in the narrow columns. Chromogens designated as *SC-9,420 artifacts* were extractable from urine with carbon tetrachloride, isooctane, and benzene; true 17-hydroxycorticosteroids were not. By paper chromatography the chromogens designated as *17-OHCS* were identifiable as cortisol, cortisone, tetrahydrocortisol, and tetrahydrocortisone; the *SC-9,420 artifacts* differed from these corticosteroids in chromatographic mobility.

Effect of a spirolactone on aldosterone secretion rate. By administering radioactive aldosterone and determining the specific activity of the aldosterone or the metabolite of aldosterone which appeared in the urine, it was possible to calculate from the degree of dilution of labeled material the amount of aldosterone which was secreted during a given period of time. Data from 2 such experiments are presented in Fig. 2 and Table I (subject J. J.). In neither study was the secretion rate of aldosterone, the urinary aldosterone level, or the urinary aldosterone metabolite level affected by treatment with the spirolactone. The fact that no significant fall in aldosterone secretion was produced by spirolactone therapy is consistent with the concept that the natriuretic action of a spirolactone is due entirely to extra-adrenal antagonism of the biologic action of aldosterone rather than to interference with the biosynthesis of aldosterone.

Effect of spirolactones on urinary 17-hydroxycorticosteroid levels. Spirolactones other than SC-9,420 had little or no effect on urinary 17-hydroxycorticosteroids (Figs. 1 and 3 and Table I). Administration of SC-9,420 (the 7α-acetyl-thio derivative) was consistently followed by a slight increase in total urinary Porter-Silber chromogens, unless special precautions were employed as described under *Methods.* The increase occurred entirely in the "free" fraction of urinary Porter-Silber chromogen; no significant alteration was detected

in the "glucuronide" fraction. That the increase in urinary Porter-Silber chromogen was indeed an artifact and not due to an alteration in metabolism or excretion of endogenous corticosteroids may be inferred from the following considerations: (1) A similar increase in urinary Porter-Silber chromogens resulted when SC-9,420 was administered to patients with Addison's disease who were excreting negligible quantities of corticosteroids. (2) The major portion of "free" Porter-Silber chromogen in the urine of subjects receiving SC-9,420 was removed by preliminary extraction with organic solvents, while the true 17-hydroxycorticosteroids and their metabolites were not (Fig. 4).

Effect of spirolactones on urine 17-ketosteroid levels. Spirolactones had no consistent effect on urinary 17-ketosteroids (Figs. 1 and 3 and Table I). In measuring 17-ketosteroids of subjects receiving SC-9,420 it was observed that a purple color interfered with quantitation of the Zimmermann reaction. By extracting the Zimmermann chromogen from the reactant solution with dichloromethane just prior to reading the optical density, it was possible to avoid this interference.

Discussion

It is well established that spirolactones are capable of reversing the effects of aldosterone on electrolyte excretion. That the natriuretic action of a spirolactone is not due to an effect on glomerular filtration rate or renal plasma flow has been established by the studies of Wiggins and colleagues.[15] It has been postulated that the mechanism of action is that of competitive inhibition[3] of the action of aldosterone at the renal tubular level. A recent report by Crabbe[16] indicates that spirolactones also inhibit the action of aldosterone at extrarenal sites. Although it has appeared certain that the natriuretic action of the spirolactones occurs mainly at a renal tubular level, there has, nevertheless, remained the possibility that spirolactones might have additional natriuretic action by inhibiting aldosterone biosynthesis within the adrenal cortex. The present study and the study of Singer[6] strongly indicate that the natriuretic action of spirolactones is not due to adrenal suppression.

The increase in aldosterone excretion which may occur during treatment with spirolactones is not to be taken as evidence that these agents have any direct stimulatory effect on adrenocortical synthesis of aldosterone. It is well established that loss of total body sodium is a physiologic stimulus to increased aldosterone secretion by the adrenals.[17, 18] Since substantial sodium loss occurred in those cases in which a rise in aldosterone output was observed, it seems reasonable to suggest that the loss of sodium rather than the spirolactone itself was responsible for the increases in aldosterone.

Many facets of aldosterone metabolism have not yet been studied, but there is no evidence from the present study to indicate that the spirolactones significantly alter the metabolism or the renal excretion of aldosterone. It appears, therefore, that the natriuretic action of the spirolactones must be explained by antagonism of the renal tubular action of aldosterone-like steroids.

The authors gratefully acknowledge the kindness of Drs. I. C. Winter and Clarence L. Gantt, of G. D. Searle and Company, Skokie, Ill., in providing the spirolactones employed in this study.

REFERENCES

1. Landau, R. L., Bergenstal, D. M., Lugibihl, K., and Kascht, M. E.: The metabolic Effects of Progesterone in Man, J. Clin. Endocrinol. **15**: 1194, 1955.
2. Cella, J. A., and Kagawa, G. M.: Steroidal Lactones, J. Am. Chem. Soc. **79**: 4808, 1957.
3. Kagawa, C. M., Cella, J. A., and Van Arman, C. G.: Action of New Steroids in Blocking Effects of Aldosterone and Desoxycorticosterone on Salt, Science **126**: 1015, 1957.
4. Liddle, G. W.: Sodium Diuresis Induced by Steroidal Antagonists of Aldosterone, Science **126**: 1016, 1957.
5. Liddle, G. W.: Aldosterone Antagonists, A. M. A. Arch. Int. Med. **102**: 998, 1958.
6. Singer, B.: Effect of Aldosterone-Antagonist, SC-8109, on the Secretion of Aldosterone in Normal Rats, Endocrinology **65**: 512, 1959.
7. Slater, J. D. H., Moxham, A., Hurter, R., and Nabarro, J. D. N.: Clinical and Metabolic Effects of Aldosterone Antagonism, Lancet **2**: 931, 1959.
8. Kliman, B., and Peterson, R. E.: Double Isotope Derivative Assay of Aldosterone in Biological Extracts, J. Biol. Chem. **235**: 1639, 1960.
9. Ulick, S., Laragh, J. H., and Lieberman, S.: The Isolation of a Urinary Metabolite of Aldosterone and Its Use to Measure the Rate of Secretion of Aldosterone by the Adrenal Cortex of Man, Tr. A. Am. Physicians **71**: 225, 1958.
10. Liddle, G. W., Richard, J. E., and Peterson, R. E.: An Improved Method for Assaying the Steroidogenic Potency of ACTH, Endocrinology **57**: 594, 1955.
11. Silber, R. H., and Porter, C. C.: The Determination of 17, 21-Dihydroxy-20-Ketosteroids in Urine and Plasma, J. Biol. Chem. **210**: 923, 1954.
12. Callow, N. H., Callow, R. K., and Emmens, C. W.: Colorimetric Determination of Substances Containing the Grouping $-CH_2 \cdot CO-$ in Urine Extracts as Indication of Androgen Content, Biochem. J. **32**: 1312, 1938.
13. Drekter, I. J., Heisler, A., Scism, G. R., Stern, S., Pearson S., and McGavack, T. H.: The Determination of Urinary Steroids. I. The Preparation of Pigment-Free Extracts and a Simplified Procedure for the Estimation of Total 17-Ketosteroids, J. Clin. Endocrinol. **12**: 55, 1952.
14. Allen, W. M.: A Simple Method for Analyzing Complicated Absorption Curves of Use in Colorimetric Determinations of Urinary Steroids, J. Clin. Endocrinol. **10**: 71, 1950.
15. Wiggins, R. A., Hutchin, M. E., Carbone, J. V., and Doolan, P. D.: Effect of Spirolactone SC-8109 on Renal Function in Normal Human Subjects, Proc. Soc. Exper. Biol. & Med. **100**: 625, 1959.
16. Crabbe, J.: Stimulation of Active Sodium Transport Across the Isolated Toad Bladder by Aldosterone in Vitro, Clin. Res. **8**: 227, 1960.
17. Luetscher, J. A., Jr., and Johnson, B. B.: The Sodium-Retaining Corticoid of Human Urine: Its Nature and Some Physiological Factors Governing Its Secretion, J. Clin. Invest. **32**: 585, 1953.
18. Duncan, L. E., Liddle, G. W., Bartter, F. C., and Buck, K.: The Effect of Changes in Body Sodium on Extracellular Fluid Volume and Aldosterone and Sodium Excretion by Normal and Edematous Men, J. Clin. Invest. **35**: 1299, 1956.

Hormone production and nitrogen excretion in paralytic acute anterior poliomyelitis

BERNT HÖKFELT, M.D., and INGMAR JUNGNER, M.D.
Stockholm, Sweden

The production of adrenocortical hormones and catechol amines, as well as thyroid function, has been studied in 8 cases of poliomyelitis with severe paralysis and the results have been correlated to the urinary excretion of total nitrogen. The analyses were made both in the acute stage and after 1 year.

The glucocorticoid production was evaluated by determination of the 17-ketogenic steroids in urine and Porter-Silber chromogens in blood. Although the output was usually inappreciably raised, a marked rise was recorded in a few cases during a short period in the initial stage of the illness. A concurrent rise was observed in the excretion of 17-ketosteroids, which was otherwise within the normal range.

The aldosterone excretion was determined in 2 cases. In one, it was found to be greatly raised during the first week after the onset of paralysis, followed by normalization, whereas in the other case all the values were normal.

The urinary excretion of free catechol amines (adrenaline and noradrenaline) was determined in 6 cases. As a rule, no marked deviation from the normal range was found. In one case, however, a greatly raised output, particularly of noradrenaline, was recorded during a few days. This was accompanied by a corresponding rise in the excretion of 3-methoxy, 4-hydroxy mandelic acid.

Thyroid function, evaluated by determination of the protein-bound iodine in serum in 6 cases, showed no appreciable deviation from the normal.

The results of the investigation give no ground for belief that the marked, lengthy negative nitrogen balance in acute paralytic poliomyelitis is induced primarily by "overproduction" of either adrenocortical or thyroid hormones.

Severe biochemical disturbances may appear in the acute stage of paralytic anterior poliomyelitis.[1, 2] In addition to acid-base imbalance—which is dependent on the changes in respiration—the chief disturbances are those of nitrogen me-

From the Department of Endocrinology, Karolinska Hospital, and the Clinical Central Laboratory, Hospital for Infectious Diseases, Stockholm, Sweden.

Supported by grants from The Swedish National Association Against Poliomyelitis and The Swedish Medical Research Council.

Received for publication Nov. 17, 1960.

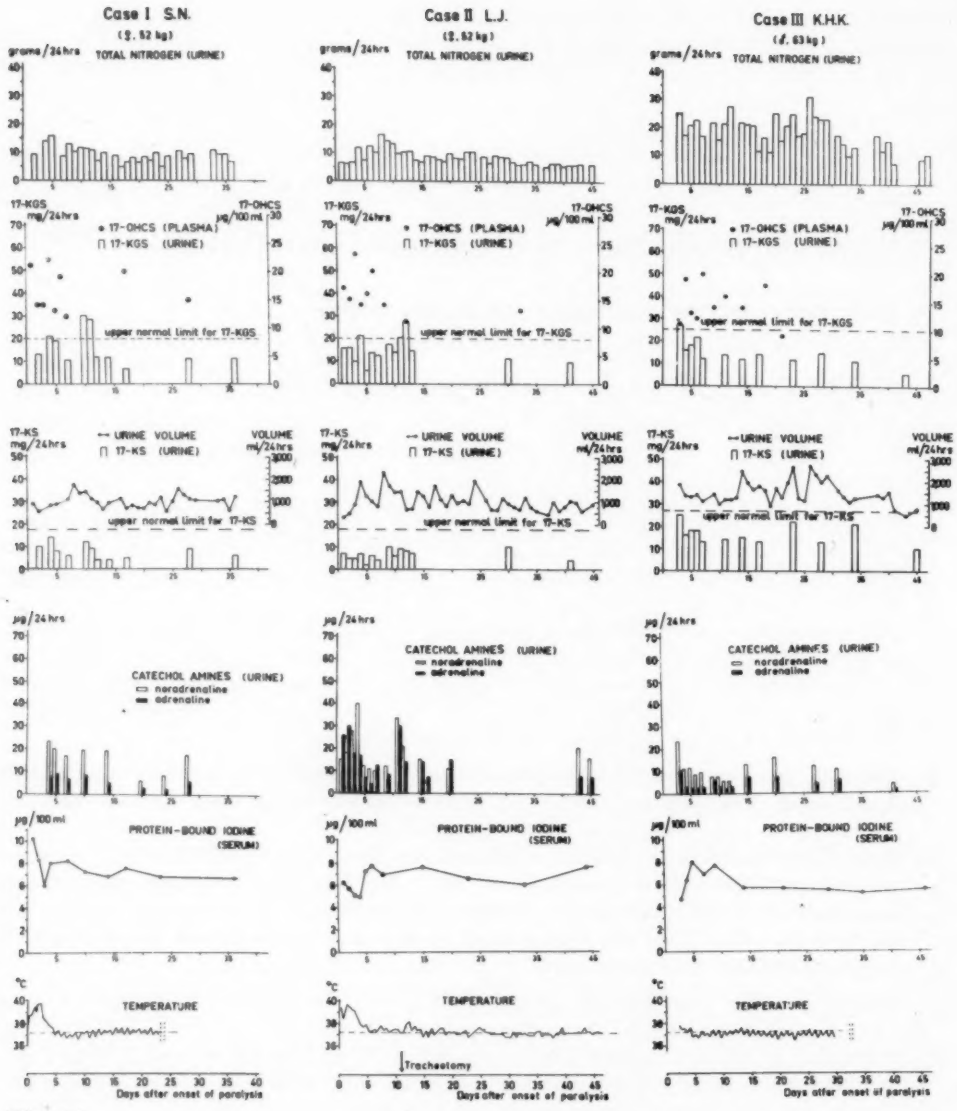


Fig. 1A.

Fig. 1. Urinary excretion of *total nitrogen*, 17-ketogenic steroids (*17-KGS*), 17-ketosteroids (*17-KS*), and catechol amines (free *adrenaline* and *noradrenaline*), plasma content of 17-hydroxy corticosteroids (*17-OHCS*), and serum protein-bound iodine (*PBI*), as well as *urine volume* and rectal *temperature*, during the acute stage of poliomyelitis in 6 cases (4 men and 2 women) of severe paralysis. *A* (above), Cases I through III; *B* (see opposite page), Cases IV through VI.

tabolism and electrolyte balance, characterized by a markedly raised urinary excretion of nitrogen, potassium, calcium, phosphate, and sulfate.[3-7] The mechanisms by which these changes take place are, however, uncertain. Diminished

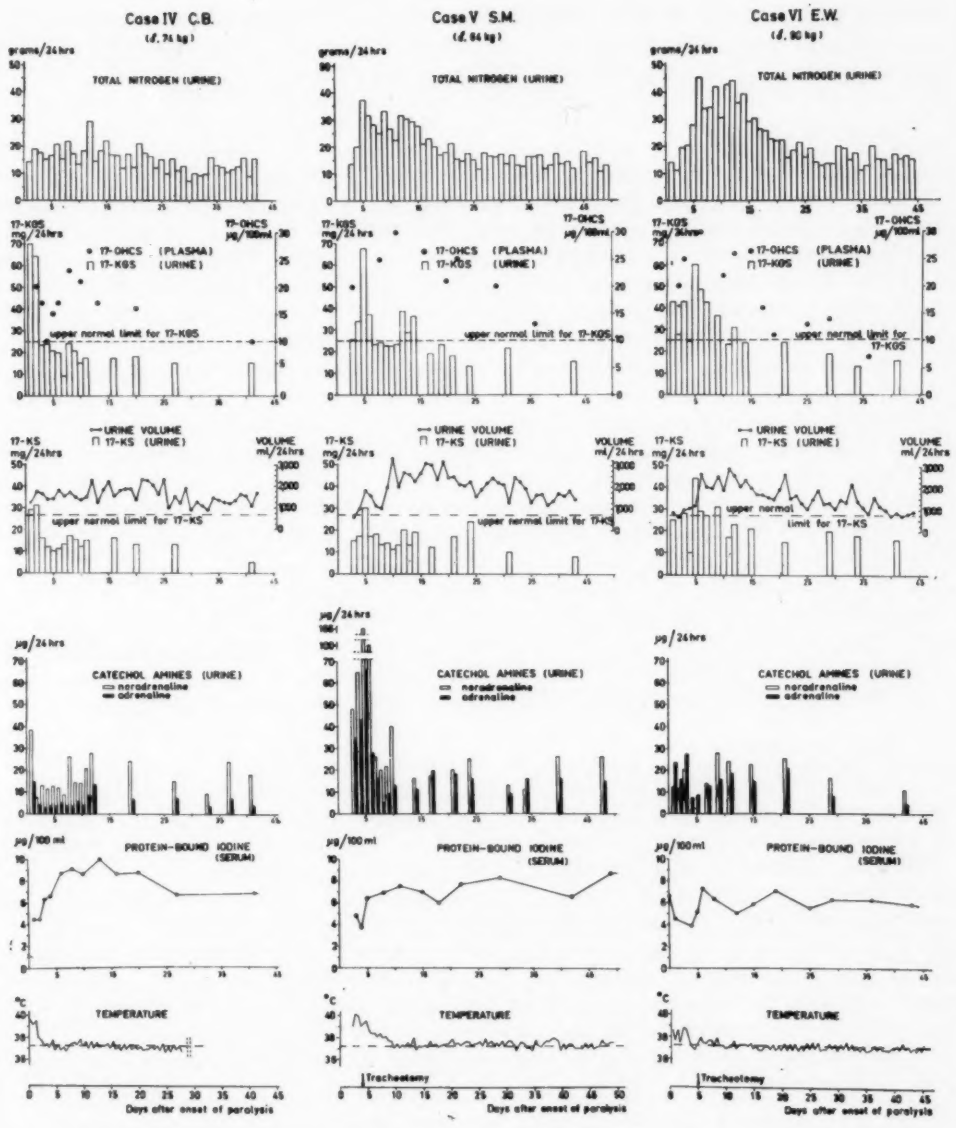


Fig. 1B. For legend see opposite page.

caloric or protein intake, fever ("toxic" destruction of protein in febrile states), "stress," immobilization, and atrophy of muscle tissue resulting from denervation have been discussed as possible causes.[4] Although various hormonal factors have been considered to play some role, only sporadic studies of endocrine function in acute poliomyelitis have been made earlier,[8-10] while systematic ones seem to be lacking. Therefore we have investigated the hormone production of the adrenal cortex, adrenal medulla, and thyroid, since these endocrine glands are intimately concerned with both nitrogen metabolism and general

stress reactions. The hormone analyses were made during the acute and earlier convalescent stage of poliomyelitis, as well as 1 year after the onset. Concurrent determinations were made of the urinary excretion of total nitrogen.

Case material

The case material comprised 8 cases of poliomyelitis (6 men and 2 women) treated at the Hospital for Infectious Diseases in Stockholm. All of them were systematically studied for a long period directly after the acute onset, which occurred in 1958 or 1959. The diagnosis was verified serologically in every case (poliomyelitis virus Type I was demonstrated in Cases I through V) and all had severe paralysis. In 3 cases (VI through VIII) paralysis was almost total in the acute stage. Tracheotomy was performed in 5 patients (II and V through VIII) and they were treated in a positive-pressure respirator for a varying period.

Cases I through IV received ordinary hospital diet, except during the initial week, when mainly carbohydrates were given; in both instances daily caloric intake varied between 1.500 and 2.500 and nitrogen intake varied between 5 and 12 Gm. per day. In Cases V and VI glucose was given intravenously initially for 3 and 4 days, respectively, supplying a minimum of 600 calories per day; thereafter they were maintained as above. In Cases VII and VIII, a short-time administration of anabolic steroids and protein hydrolysate was performed, making an evaluation of nitrogen balance impossible. Adequate fluid intake was supplied in relation to frequent determinations of the electrolyte and fluid balance.

The determinations of total nitrogen refer to urine only. The quantity of nitrogen administered was not recorded. The 24 hour urinary output was collected without preservative and, when the analysis were not made immediately, was kept cold. The investigation was counted as starting on the day of onset of paralysis (day zero).

The salient data in the 8 cases are given in the following section.

Case reports

Case I, Patient S. N. (Case sheet 4603/58). The patient was a 34-year-old female office worker who had earlier been healthy. The onset occurred November 21, 1958, in the form of headache and back pain. She was hospitalized on November 23 with aseptic meningitis (neutrophil pleocytosis). On November 24, there was incipient paralysis of the muscles of the trunk and lower limbs (day zero). She was hyperpyretic. On November 25 and 26, the paralysis extended to the muscles of the back and both shoulders, hips, and legs. From November 27 to December 5 she was placed in a cuirass respirator (Freiberger type). She had no bulbar symptoms or signs of encephalitis. She was discharged on June 1, 1959, with residual spinal paralysis; it was slight in the arms and shoulders and moderate in the trunk, right leg, and right hip, whereas the left leg and hip were almost totally paralyzed.

Her weight on admission was 52 kilograms, after 12 weeks, 47 kilograms, and after 1 year, 53 kilograms.

Case II, Patient L. E. J. (Case sheet 4619/58). The patient was a 23-year-old female office worker who had earlier been healthy. The onset took place December 6, 1958, in the form of fever and headache. Neck rigidity and incipient weakness of the legs appeared on December 7 (day zero). She was hospitalized on the same day with aseptic meningitis (neutrophil pleocytosis). On December 8, there were signs of spinal respiratory paralysis. Although she was treated in a cuirass respirator (Freiberger type) from December 9 through 18, she was still somewhat underventilated. Paralysis progressed in the legs, trunk, and arms. She had no bulbar symptoms or signs of encephalitis. On December 18 an acute exacerbation occurred, necessitating tracheotomy and positive-pressure respiration (Engström respirator). Her condition subsequently improved, but extensive spinal paralysis persisted. She was decannulated on August 5, 1959. At the time of writing (June, 1960), the patient still needs a cuirass respirator periodically. She has residual spinal paralysis; it is moderate to severe in the arms and trunk, and almost total in the hips and lower limbs.

Her weight on admission was 52 kilograms, after 12 weeks, 43 kilograms, and after 1 year, 40 kilograms.

Case III, Patient K. H. K. (Case sheet 4550/58). The patient was a 25-year-old policeman who had earlier been healthy. The onset occurred November 13, 1958, in the form of headache and fever. On November 17, neck rigidity as well as weakness of the arms and legs appeared, and the patient was unable to urinate (day zero). He was hospitalized November 19 with aseptic meningitis (lymphocytic pleocytosis) and slight to moderate spinal paralysis of practically all muscle groups. He had no bulbar symptoms or signs of encephalitis. He was subfebrile for 3 days, after which rapid general improvement took place, although paralysis continued to extend, particularly to the muscles of the arms, shoulders, and trunk. He was discharged on February 2, 1959, with moderate residual spinal paralysis, chiefly of the right arm and leg. The patient (using a surgical corset) resumed full-time work in June, 1960, but the paralysis has remained largely unchanged.

His weight on admission was 73 kilograms, after 12 weeks, 62 kilograms, and after 1 year, 64 kilograms.

Case IV, Patient C. B. (Case sheet 4386/58). The patient was a 31-year-old businessman who had earlier been healthy. The onset took place November 8, 1958, in the form of aches in the chest and back. On November 11 he was hyperpyretic; neck rigidity was present, as well as incipient weakness of the right leg (day zero). He was hospitalized on the same day with aseptic meningitis (lymphocytic pleocytosis), paralysis of the right hip and leg, and urinary retention. He had no respiratory paralysis, no bulbar symptoms, and no signs of encephalitis. He became afebrile on November 14. There was residual paralysis of the right leg and hip, very severe paralysis of the left leg and hip, and moderate paralysis of the trunk muscles. He was discharged on June 22, 1959, without appreciable regression of the paralysis.

His weight on admission was 74 kilograms, after 12 weeks, 69 kilograms, and after 1 year, 72 kilograms.

Case V, Patient S. M. (Case sheet 4069/58). The patient was a 20-year-old lumberman who had earlier been healthy. The onset took place October 23, 1958, in the form of fever, pain in the head, back, and nape of neck, and incipient weakness of the legs (day zero). He was hospitalized October 25 with high fever, systemic disturbances, and aseptic meningitis (lymphocytic pleocytosis). He had extensive, severe spinal paralysis, high cervical myelitis, and spinal dysphagia. He was placed in a cuirass respirator (Freiberger type) for the next few days. On October 27, tracheotomy was performed because of stagnation of secretion in the throat and rising blood pressure; positive-pressure respiration was instituted with an Engström respirator. His general condition improved, but he was still febrile during the next few days. Respiratory and swallowing function became normal. There was residual paralysis of all muscle groups; it was generally moderate, but was most severe in the shoulders, hips, and left leg. He was decannulated on February 19, 1959. On discharge on June 2, 1959, there was no appreciable regression of the paralysis.

His weight on admission was 64 kilograms, after 12 weeks, 61 kilograms, and after 1 year, 67 kilograms.

Case VI, Patient E. W. (Case sheet 4202/58). The patient was a 30-year-old male engineer who had earlier been healthy. The onset occurred October 29, 1958, in the form of headache, neck rigidity, fever, and pain in the legs. He was hospitalized November 2 with aseptic meningitis (pleocytosis: neutrophils 50 per cent, lymphocytes 50 per cent), and paralysis of the leg and trunk muscles (day zero). Within about 1 day, practically total paralysis appeared, including spinal respiratory paralysis. He was placed in a cuirass respirator (first of Freiberger type, then a Sahlin respirator). On November 6, his general condition became affected; there was stagnation of secretion in the throat and decreased oxygen saturation of the blood. He was also "encephalitic." On December 7, tracheotomy was performed, and positive-pressure respiration instituted with an Engström respirator. This produced an improvement in his general condition. He was decannulated on October 6,

1959. At the time of writing (June, 1960), the patient is still in a cuirass respirator. He has severe residual paralysis of the arms and trunk, and total paralysis from the hips downward.

His weight on admission was 90 kilograms, after 12 weeks, 68 kilograms, after 1 year, 61 kilograms.

Case VII, Patient H. Å. T. (Case sheet 264/59). The patient was a 23-year-old male student who had been hospitalized in 1948 with an illness diagnosed as nonparalytic poliomyelitis. The onset took place January 13, 1959, in the form of fever, headache, and neck rigidity. He was hospitalized January 14, with systemic disturbances and aseptic meningitis (neutrophil pleocytosis). Incipient flaccid paralysis of the legs appeared on January 15 (day zero). He was hyperirritable and "encephalitic." On January 17 he was hyperpyretic and had generalized paralysis, including respiratory paralysis and bilateral pharyngeal paresis (with the so-called "angle symptom"[11]). Later in the same day, he suddenly became cyanotic and pulseless, necessitating tracheotomy and positive-pressure respiration (Engström respirator). Thereafter, his general condition improved rapidly. He was decannulated on March 1, 1960. At the time of writing (June, 1960), the patient is still dependent on a cuirass respirator. Residual paralysis is severe in the arms, shoulders, and trunk, and he is totally paralyzed from the hips downward.

His weight on admission was 62 kilograms, after 12 weeks, 49 kilograms, after 1 year, 46 kilograms.

Case VIII, Patient S. L. N. (Case sheet 281/59). The patient was a 23-year-old builder who had earlier been healthy, except for recurrent peptic ulcer. The onset took place January 12, 1959, in the form of headache, neck rigidity, and fever. On January 14, weakness of the arms and legs appeared; he had difficulty in breathing and coughing, and was unable to urinate (day zero). He was hospitalized on the same evening, when he was hyperpyretic with systemic disturbances, aseptic meningitis (lymphocytic pleocytosis), and generalized spinal paralysis. Although he was placed in a cuirass respirator (Freiberger type), his general condition deteriorated. Pharyngeal paralysis appeared, and he was "encephalitic." On January 16, tracheotomy was performed, and he was placed in an Engström respirator, which resulted in rapid improvement in his general condition. At the time of writing (June, 1960), the patient is still in a respirator. He has almost total residual spinal paralysis of the left arm and shoulder, trunk, hips, and right leg, and moderate paralysis of the right arm and left leg.

His weight on admission was 62 kilograms, after 12 weeks, 42 kilograms, after 1 year, 39 kilograms.

At examination one year after onset, 2 patients (Cases VII and VIII) had somewhat more abundant body hair and beard than earlier, whereas none of the other patients showed any clinical signs that might indicate changes in endocrine function.

Methods*

Adrenocortical function was evaluated on the basis of the following steroid hormone analyses. Normal values given refer to figures obtained in the laboratory of one of the authors from studies of normal individuals aged 20 to 50 years.

In urine, 17-ketogenic steroids (17-KGS) were determined as total 17-hydroxy steroids by the method of Appleby and Norymberski.[12] Normal mean value: 14.2 (range, 8 to 25) mg. per 24 hours in men and 12.1 (range, 5 to 20) mg. per 24 hours in women.

In urine, 17-ketosteroids (17-KS) were determined according to Zimmermann by the modification of Vestergaard.[13] Normal mean value: 15.5 (range, 8 to 27) mg. per 24 hours in men and 9.8 (range, 4 to 18) mg. per 24 hours in women.

*The steroid analyses were carried out in the laboratories of one of us (B. H.), the PBI determinations were made at the Clinical Laboratories, Centrallasattet, Danderyd, Sweden, and the remaining laboratory investigations were performed in the laboratories of the other author (I. J.).

In blood, 17-hydroxy corticosteroids (17-OHCS) were determined as Porter-Silber chromogens according to Eik-Nes.[14] Normal range: 5 to 15 μg per 100 ml. of plasma (morning value) in both men and women.

Aldosterone in urine was determined according to Neher and Wettstein[15] in the modification of Mattox and Lewbart.[16] Normal range: 1 to 9 μg per 24 hours in both men and women.

The production of catechol amines in the organism was followed by determining the urinary excretion of free adrenaline and noradrenaline; in a few cases, the excretion of 3-methoxy, 4-hydroxy mandelic acid was also determined.

Adrenaline (A) and noradrenaline (NA) in urine were determined according to Euler and Floding.[17] Normal range (in recumbency): A, 2 to 4 μg per 24 hours; NA, 10 to 20 μg per 24 hours.

In urine, 3-methoxy, 4-hydroxy mandelic acid (MHMA) was determined according to Sandler and Ruthven.[18, 19] Normal value: 5.8 (range 1.9 to 7.1) mg. per 24 hours.

Thyroid function was evaluated by determining the protein-bound iodine (PBI) in serum according to Barker and associates[20] by the modification of Widström and Björkman.[21] Normal range: 4 to 8 μg per 100 ml. plasma in both men and women.

Total nitrogen in urine was determined by the Kjeldahl technique.

Hematocrit was determined according to Hedin in the modified technique of Norberg and Warvenius.[22] Normal range: 40 to 54 per cent in men, 37 to 47 per cent in women.

The body temperature was measured rectally both morning and afternoon.

Results

Acute stage.

STEROID ANALYSES. It can be inferred from Figs. 1 and 2 that frequent determinations of 17-KGS and 17-KS in urine, as well as of 17-OHCS in blood,

Table I. Aldosterone excretion in 2 cases of paralytic poliomyelitis (Cases V and VI)

Case	*Day after onset of paralysis*	*Aldosterone (μg/24 hours)*	*Hematocrit (%)*
V, Patient S. M. (♂)	4	20	50
	5	40	32
	6-7	48*	24
	8	16	37
	9-10	20*	
	11	3.0	36
	14-15	6.0*	33
	47-48	3.6*	47
	1 year	2.4	
	1 year	2.4	
	1 year	2.5	
VI, Patient E. W. (♂)	3-4	2.2*	43
	5	6.3	48
	9	< 9.0	37
	13	< 3.0	40
	14-15	4.0*	40
	21	< 4.0	38
	42-43	5.5*	42

*Determined on 48 hour urine specimen.

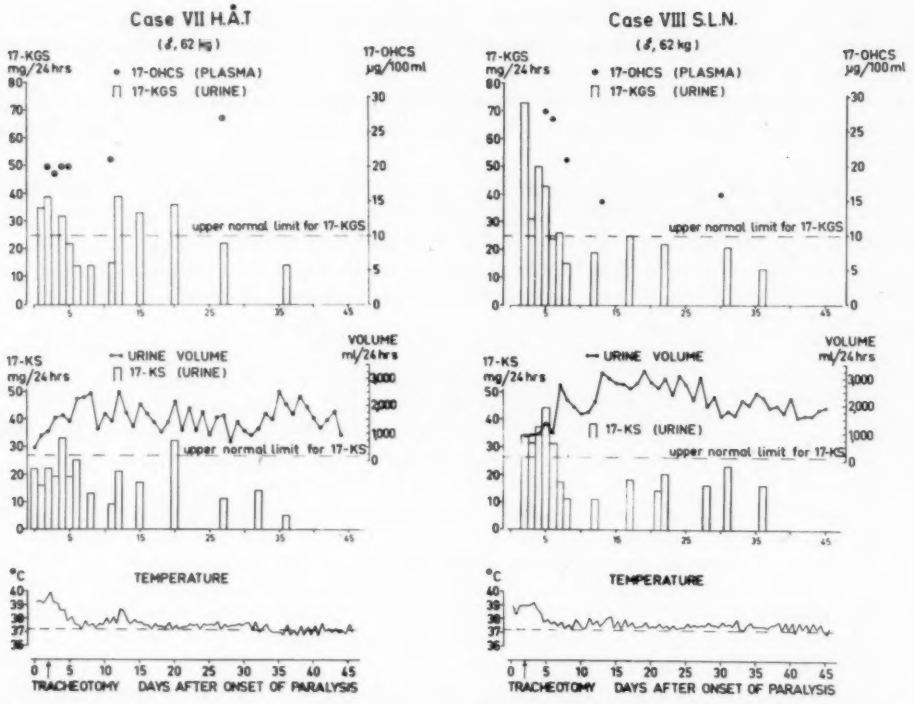


Fig. 2. Urinary excretion of 17-ketogenic steroids (*17-KGS*) and 17-ketosteroids (*17-KS*), and plasma content of 17-hydroxy corticosteroids (*17-OHCS*), as well as *urine volume* and rectal *temperature*, during the acute stage of poliomyelitis in 2 men with severe paralysis (Cases VII and VIII).

were made in all the patients during the first 45 days after the onset of paralysis. The results of both blood and urine analyses indicate that an increased production of corticosteroids takes place in every case on some occasion, although the size and duration of the rise vary greatly. Despite the fact that all were relatively severe cases, 3 of them (Cases I through III) had only inappreciably raised values in both blood and urine. It is true that greatly raised urinary values (about 60 to 70 mg. 17-KGS per 24 hours) were recorded in 4 cases (IV through VI, and VIII), including some of the most severe ones. This marked rise had, however, a duration of only one or a few days, and was followed by moderately raised values (30 to 40 mg. 17-KGS per 24 hours) for 1 to 2 weeks. In one severe case (VII) moderately increased adrenocortical activity with raised values was still present on the twentieth day. In every case the values were, however, within the normal range from the fourth week after the onset of paralysis and onward. It can be mentioned that in all 5 patients who were tracheotomized, the highest KGS values were recorded in connection herewith.

The excretion of 17-KS ran largely parallel with the values for 17-KGS. In 2 cases (VI and VIII), 17-KS values exceeding 40 mg. per 24 hours were recorded.

Analyses of aldosterone in urine could be made in 2 patients only (Cases V

and VI); the results are given in Table I. In one of them (Case V) a considerably raised aldosterone output (20 μg per 24 hours) was already present at the first determination, performed on the fourth day after the onset of paralysis. This was followed by a further rise to 48 μg per 24 hours on the sixth and seventh day, after which the values fell, and reached the normal level after another few days. In the other case (VI) the aldosterone excretion was normal, the values ranging from 2.2 to 9.0 μg per 24 hours.

CATECHOL AMINES. The urinary excretion of cathechol amines was determined in cases I through VI. It is seen from Fig. 1 that the excretion of free adrenaline was normal or only inappreciably raised in the less severe cases (I, III, and IV). In the more severe cases—in which a respirator was required (Cases II, V, and VI)—the output was, on the other hand, moderately or even greatly raised, a peak value of 68 μg per 24 hours being recorded in one case (V). The raised adrenaline excretion was of short duration in all but Case V, in which an increased value was recorded as late as 45 days after the onset of paralysis.

For free noradrenaline normal or only slightly raised values generally were recorded. In one case (V) the output was, however, greatly increased; after a rise to about 166 μg per 24 hours, the values rapidly became normal. The maximal excretion of noradrenaline coincided with that of adrenaline.

The determinations of 3-methoxy, 4-hydroxy mandelic acid (MHMA) in urine made in 3 cases (IV through VI) showed good agreement with the values for free catechol amines. Thus, the patient (V) who had the highest output of free catechol amines (totally 233 μg per 24 hours) showed a maximal MHMA excretion of 22.5 mg. per 24 hours (Fig. 3). Moreover, the MHMA values were found to lie within the normal range in 2 patients with normal excretion of free catechol amines.

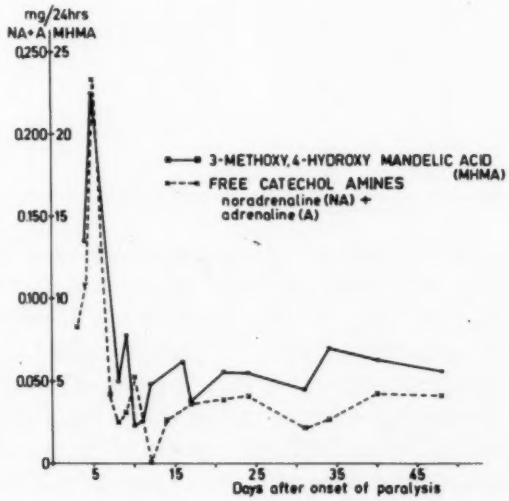


Fig. 3. Urinary excretion of *3-methoxy, 4-hydroxy mandelic acid* and *free catechol amines* (noradrenaline plus adrenaline) during the acute phase of poliomyelitis in 1 case of severe paralysis (Case V, Patient S. M.).

Table II. Urinary excretion of total nitrogen, 17-ketosteroids (17-KS), 17-ketogenic steroids (17-KGS), free adrenaline (A), free noradrenaline (NA), 3-methoxy, 4-hydroxy mandelic acid (MHMA), and protein-bound iodine (PBI) in serum: Determinations 1 year after onset

Case	*Total N (Gm./ 24 hours)*	*17-KS (mg./ 24 hours)*	*17-KGS (mg./24 hours)*	*A (μg/24 hours)*	*NA (μg/24 hours)*	*MHMA (mg./24 hours)*	*PBI (μg/ 100 ml.)*
I, Patient S. N.	5.6	12	18				
(♀)	6.6	7	11	3	13	3.3	5.8
	7.6	9	6				
	6.6	7	7				
II, Patient L. J.	8.3	5	14				
(♀)	5.9	4	4	5	14	3.7	6.0
	7.9	6	6				
III, Patient K. H. K.	17.8	23	30				
(♂)	16.1	18	19	7	8	5.5	5.6
	15.8	17	24				
IV, Patient C. B.	9.7	12	17	6	11	6.2	8.0
(♂)	12.5	14	15				
V, Patient S. M.	10.0	10	10				
(♂)	11.8	12	18	8	10	3.7	5.8
	7.7	11	14				
VI, Patient E. W.	9.1	19	31				
(♂)	8.9	20	28	6	19	3.6	5.9
	11.1	27	34				
	6.6	17	29				
VII, Patient H. Å. T.	5.6	13	12				
(♂)	6.5	13	24	2	10	5.5	6.2
	8.0	14	13				
VIII, Patient S. L. N.	5.8	11	10				
(♂)	4.4	9	11	2	4	3.4	7.2
	8.9	14	16				
	5.8	11	14				

PROTEIN-BOUND IODINE IN SERUM. Judging by the 6 cases in which the PBI was determined (Fig. 1), the values showed a tendency to fall towards the lower normal limit (4 μg per 100 ml.) during the first few days after the onset of paralysis, possibly corresponding to the period of raised body temperature. After this time, the PBI lay entirely within the normal range (4 to 8 μg per 100 ml.) in 4 cases. Occasional, slightly raised values were observed in Case V, whereas in Case IV several raised values (up to 10 μg per 100 ml.) were recorded during the fifth to the twentieth day. Clinically, none of the patients showed any signs of hyperthyroidism.

TOTAL NITROGEN IN URINE. The urinary excretion of nitrogen normally varies in relation to protein intake and body size. The high carbohydrate but low

protein standard diet given to our patients with poliomyelitis during the acute stage normally results in a nitrogen excretion of 5 to 10 Gm. per 24 hours. An excretion in excess of this quantity is a measure of muscle destruction.

In paralytic poliomyelitis, the nitrogen excretion usually has a characteristic course, with a maximum around the fifth to tenth day after the onset of paralysis, followed by a continuous negative nitrogen balance for months on end. This applied in the present series (Fig. 1). The initial nitrogen loss was particularly evident in the most severe cases (V and VI), in which paralysis progressed very rapidly and affected all groups of muscles at the same time. In such cases, the excretion may amount to 30 to 50 Gm. N per 24 hours, representing destruction of ½ to 1 Kg. of wet muscle per 24 hours. In cases where clinical spreading of paralysis occurs more slowly—as was especially noticeable in Case III—this is, in fact, reflected in a more protracted course of nitrogen excretion. The total nitrogen excretion in the present series ranged from 350 to 950 Gm. during the first 6 weeks,[7] 150 to 500 Gm. of this quantity presumably representing protein breakdown.

A peak in nitrogen excretion was observed in connection with tracheotomy in cases V and VI, whereas this did not apply in the other 3 tracheotomized patients.

The urine volume varied greatly in the different cases (Figs. 1 and 2). It was, however, usually low to normal during the first week after the onset, moderately raised (to 2,000 to 3,000 ml. per 24 hours) in the second or third week—parallel to the maximal excretion of total nitrogen—with a return to normal volume from the fifth week at the latest.

BODY TEMPERATURE. The body temperature was initially raised in every case (Figs. 1 and 2), but usually became normalized within a week.

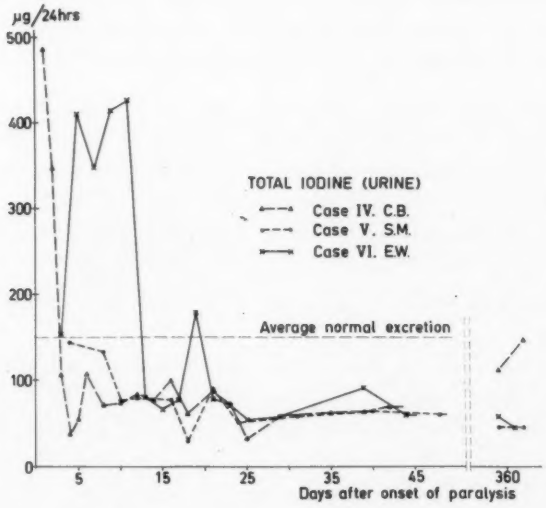


Fig. 4. Urinary excretion of total iodine in the acute phase of poliomyelitis: 3 cases of severe paralysis.

One year after onset of paralysis. All the values obtained in the relevant hormone analyses made 1 year after the onset of paralysis are collected in Table II. On the whole, the urinary excretion of 17-KGS and 17-KS was normal. In Case III a somewhat high steroid output was, however, present in a single determination, and in Case VI slightly raised values were recorded in 4 analyses. The urinary excretion of total nitrogen was within the normal range in all except Case III, in which it was somewhat high.

New determinations were made after a further 6 months. On this occasion, both Case III and Case VI had normal steroid and nitrogen excretion (mean values for 17-KGS were 21.3 and 15.8 mg. per 24 hours, respectively, and for total nitrogen values were 11.1 and 6.7 Gm. per 24 hours).

It is also evident from Table II that the values for adrenaline, noradrenaline, MHMA, and PBI lay within the normal range.

Discussion

Whedon and Shorr[4] demonstrated, in systematic metabolic studies, that acute paralytic poliomyelitis is associated with an extraordinarily great and lengthy loss of nitrogen. The loss was larger (200 to 400 Gm. total accumulated nitrogen balance), lasted longer (2 to 5 months), and showed a peak later (sixteenth day after first symptoms of disease) than has been found to apply in other conditions accompanied by protein depletion, e.g., infections, fractures, and surgical operations.[23-25] Against the background of earlier studies in healthy men,[26] they considered that at most 25 to 30 per cent of the total protein loss in poliomyelitis patients could be ascribed to immobilization. The remainder was regarded to depend on other factors, chiefly muscle atrophy, as a result of denervation. The main object of the present investigation was to ascertain whether any correlation could be demonstrated between the nitrogen loss in acute poliomyelitis and the hormonal production of the adrenal cortex, adrenal medulla, and thyroid gland. This was to obtain an idea of the possible implication of these hormones in the breakdown of protein.

That an excess of glucocorticoids can lead to a markedly negative nitrogen balance is illustrated in Cushing's disease, as well as by the results of metabolic studies with administration of cortisone. However, this requires large quantities of cortisone over a long period. Thus, a daily dose of 200 mg. for 24 days was found to lead to a nitrogen loss of about 104 Gm., and the same dose given for 76 days resulted in a loss of about 302 Gm. of nitrogen,[27-28] whereas a dose of 12 mg. of cortisone per hour for 8 hours produced no demonstrable increase in nitrogen excretion.[29]

Our investigations show that some activation of adrenocortical function takes place in an early stage of poliomyelitis, but that it is of relatively short duration, and is only infrequently of any magnitude. Assuming that the 17-KGS values represent at least 60 per cent of the total glucocorticoid production, the adrenal secretion on a single occasion can be calculated to have amounted to maximally 120 mg. of hydrocortisone per 24 hours, whereas, during the incomparably greater part of the time, it lay within the normal range, i.e., 16 to 26 mg. per 24 hours.[30] It is unlikely that the comparatively moderate gluco-

corticoid production in acute poliomyelitis, which is of the same order of magnitude as that seen in trauma, surgical interventions, and other stresses on the organism,[31-33] would have made any noteworthy contribution per se to the nitrogen loss of up to 500 Gm. during a 45 day period recorded in our series. It is possible however, that the peak in nitrogen excretion often seen in the initial stage of poliomyelitis is to be ascribed partly to increased adrenocortical activity. The role of the glucocorticoids seems likely to be of a "permissive" nature, and not that of primary cause of the nitrogen waste.

The serum content of PBI is generally regarded as a good index of thyroid function. In poliomyelitis, the iodine released in connection with the great muscle breakdown during the acute paralytic stage is a possible source of interference. The urinary excretion of free iodine in Cases IV and VI (Fig. 4) was determined,* therefore, using the method of Widström and Claeson.[34] It is seen that, even in connection with marked muscle breakdown, the excretion is only moderately raised, and for a short time (up to 500 μg per 24 hours for 2 to 14 days), whereas, generally it is normal, i.e., about 150 μg per 24 hours.[35] Consequently, these quantities of iodine could have had only an inappreciable effect on the PBI values, either directly or indirectly, via thyroid inhibition. Judging by the values for PBI recorded in our cases, an overproduction of thyroid hormone cannot be the direct cause of protein breakdown.

Consequently, it does not seem probable that the poliomyelitis virus—despite its special affinity to the brain stem—has any greater stimulatory influence on the centers which, via the pituitary, regulate the hormone production of the adrenal cortex and the thyroid gland than have illnesses and injuries in general. The affinity of the poliomyelitis virus to the brain stem could be envisaged as leading to destruction of certain centers in this region, with secondary adrenocortical insufficiency as a result. Even if this does not seem to have applied to any of the patients in the present series, this possibility should be borne in mind in the presence of incipient falling blood pressure and circulatory and renal failure, these conditions having been found to yield a high mortality rate.[36]

Disturbances in electrolyte metabolism are not uncommon in paralytic poliomyelitis,[1-3, 6, 37] and considerable hypokaliemia is often present. Since aldosterone—the most potent electrolyte-regulating hormone of the adrenal cortex—can produce raised potassium excretion and hypokaliemia, aldosterone determinations are of particular interest in this connection. Although our analyses were limited to 2 cases, they showed that a rise in glucocorticoid production may be accompanied by a greatly raised excretion of aldosterone, but that the opposite could also apply. In one case, the urinary aldosterone output during a few days increased to almost 50 μg per 24 hours. This should represent a secretion of maximally 1 mg. of aldosterone per 24 hours, as compared to the normal of about 0.2 mg.[38] During this time the serum potassium was, however, within normal limits (5.0 to 4.3 mEq. per liter). This is in good agreement with the finding that administration of 0.5 to 3 mg. of aldosterone per 24 hours for up to 14 days had only an inappreciable effect on the potassium content of the

*We are indebted to Dr. G. Widström for carrying out these determinations.

blood, despite a considerable rise in the urinary excretion of potassium.[39, 40] At a later stage of the illness, i.e., during the time when hypokaliemia would have been apt to appear, the aldosterone excretion was normal in both cases. This argues against aldosterone playing any essential role in the development of hypokaliemia in paralytic poliomyelitis.

The cause of the raised aldosterone production observed by us is uncertain. Dehydration, with a resulting decrease in extracellular volume, generally leads to a rise in aldosterone production.[41] Judging by the hematocrit determinations made (Table I), this mechanism does not, however, seem to have been decisive in the case in question. A possible explanation is that the virus infection led to a specific effect on the cerebral centers that regulate the adrenal production of aldosterone.[42] The secretion of aldosterone does not seem to be under the direct influence of the anterior lobe of the pituitary. It is nevertheless possible that raised ACTH secretion may be of importance, since under certain conditions ACTH has been found to influence aldosterone secretion.[43]

Marked stress is usually considered to influence not only the adrenal cortex, but also the adrenal medulla and sympathetic nervous system, an observation that is borne out by the present studies of the urinary excretion of free catechol amines and MHMA. Noradrenaline was found to be predominant, as compared to adrenaline, in the cases in which the catechol amine output was raised, a finding similar to the observations in surgical stress and allied conditions.[32] The implication of this proportionately greater rise in noradrenaline excretion—as compared to that of adrenaline—is not clear. In view of the concurrent activation of the adrenal cortex, it could perhaps be expected that the noradrenaline output would be proportionately less affected than the adrenaline output. Luft and Euler[44] found, in fact, that administration of both ACTH and cortisone led to a diminished urinary excretion of noradrenaline. It is therefore evident that further studies should be made of the mutual relation between adrenocortical and adrenomedullary function under conditions of stress.

REFERENCES

1. Bower, A. G., Chudnoff, J. S., and Chaney, A. L.: Biochemical Alterations in Acute Human Poliomyelitis: Electrolyte Patterns and Trends, California Med. 73: 406, 1950.
2. Jungner, I.: Poliomyelitis: Papers and Discussions Presented at the Fourth International Poliomyelitis Conference, Philadelphia, 1958, J. B. Lippincott Co., p. 530.
3. Bower, A. G., Morgan, F. M., and Chaney, A. L.: Nitrogen and Potassium Metabolism: the Reaction Pattern in Poliomyelitis, Am. J. M. Sc. 223: 532, 1952.
4. Whedon, G. D., and Shorr, E.: Metabolic Studies in Paralytic Acute Anterior Poliomyelitis. I. Alterations in Nitrogen and Creatine Metabolism, J. Clin. Invest. 36: 941, 1957.
5. Whedon, G. D., and Shorr, E.: Metabolic Studies in Paralytic Acute Anterior Poliomyelitis. II. Alterations in Calcium and Phosphorus Metabolism, J. Clin. Invest. 36: 966, 1957.
6. Whedon, G. D., and Shorr, E.: Metabolic Studies in Paralytic Acute Anterior Poliomyelitis. IV. Effects of Testosterone Propionate and Estradiol Benzoate on Calcium, Phosphorus, Nitrogen, Creatine and Electrolyte Metabolism, J. Clin. Invest. 36: 995, 1957.

7. Jungner, I., and Jungner, G.: Disturbances in Nitrogen Metabolism in the Acute Stage of Paralytic Poliomyelitis, Acta med. scandinav. **168**: 169, 1960.
8. Luft, R., and Müller, R.: Endocrine and Mental Disorders in Acute Anterior Poliomyelitis, Acta med. scandinav. **127**: 448, 1947.
9. Gemzell, C. A.: Adrenal Cortical Activity of Patients With Poliomyelitis Acuta. Acta med. scandinav. **154**: 104, 1956.
10. Allott, E. N.: Hypernatraemia and Hyperchloraemia in Bulbar Poliomyelitis, Lancet **1**: 246, 1957.
11. Sjöberg, A.: Respiratory Obstruction in Spinobulbar Poliomyelitis, Acta oto-laryng. **46**: 153, 1956.
12. Appleby, J. I., and Norymberski, J. K.: Urinary Excretion of 17-Hydroxy-20-Oxosteroids in Normal Health and in Rheumatoid Arthritis, Ann. Rheumat. Dis. **14**: 172, 1955.
13. Vestergaard, P.: Rapid Micro-Modification of the Zimmermann-Callow Procedure for the Determination of 17-Ketosteroids in Urine, Acta endocrinol. **8**: 193, 1951.
14. Eik-Nes, K.: Determination of 17, 21-Dihydroxy-20-Ketosteroids in Blood Plasma, J. Clin. Endocrinol. **17**: 502, 1957.
15. Neher, R., and Wettstein, A.: Physiochemical Estimation of Aldosterone in Urine, J. Clin. Invest. **35**: 800, 1956.
16. Mattox, V. R., and Lewbart, M. L.: The Determination of Aldosterone in Urine, J. Clin. Endocrinol. **19**: 1151, 1959.
17. Von Euler, U. S., and Floding, I.: Diagnosis of Pheochromocytoma by Fluorimetric Estimation of Adrenaline and Noradrenaline in Urine, Scandinav. J. Clin. & Lab. Invest. **8**: 288, 1956.
18. Sandler, M., and Ruthven, C. R. J.: Quantitative Colorimetric Method for Estimation of 3-Methoxy-4-Hydroxymandelic Acid in Urine, Lancet **2**: 114, 1959.
19. Sandler, M., and Ruthven, C. R. J.: Colorimetric Estimation of 3-Methoxy-4-Hydroxymandelic Acid in Urine, Lancet **2**: 1034, 1959.
20. Barker, S. B., Humphrey, M. J., and Soley, M. H.: The Clinical Determination of Protein-Bound Iodine, J. Clin. Invest. **30**: 55, 1951.
21. Widström, G., and Björkman, M.: Alkaline Incineration of Iodine-Containing Organic Material, Acta chem. scandinav. **12**: 1881, 1958.
22. Norberg, B., and Warvenius, S.: A Hematocrit Centrifuge, Scandinav. J. Clin. & Lab. Invest. **4**: 249, 1952.
23. Grossman, C. M., Sappington, T. S., Burrows, B. A., Lavietes, P. H., and Peters, J. P.: Nitrogen Metabolism in Acute Infections, J. Clin. Invest. **24**: 523, 1945.
24. Howard, J. E., Parson, W., Stein, K. E., Eisenberg, H., and Reidt, V.: Studies on Fracture Convalescence. I. Nitrogen Metabolism After Fracture and Skeletal Operations in Healthy Males, Bull. Johns Hopkins Hosp. **75**: 156, 1944.
25. Moore, F. D., and Ball, M. R.: The Metabolic Response to Surgery, Springfield, 1952, Charles C Thomas, Publisher.
26. Deitrick, J. E., Whedon, G. D., and Shorr, E.: Effects of Immobilization Upon Various Metabolic and Physiologic Functions of Normal Men, Am. J. Med. **4**: 3, 1948.
27. Luft, R., and Sjögren, B.: Metabolic Studies With Cortisone, Acta endocrinol. **7**: 211, 1951.
28. Sjögren, B.: Studies on the Effect of 11-Desoxy-Corticosterone Acetate (DCA), 11-Dehydro-17-Hydroxy-Corticosterone Acetate (Cortison Acetate) and Adrenocorticotropic Hormone (ACTH) on the Metabolism of Sodium, Chloride and Potassium, Thesis, University of Stockholm, 1952.
29. Thorn, G. W., Jenkin, D., and Laidlaw, J. C.: The Adrenal Response to Stress in Man, Recent Progr. Hormone Res. **8**: 471, 1953.
30. Peterson, R. E., and Wyngaarden, J. B.: The Miscible Pool and Turnover Rate of Hydrocortisone in Man, J. Clin. Invest. **34**: 957, 1957.

31. Conn, J. W., Fajans, S. S., Louis, L. H., Seltzer, S. H., and Kaine, H. D.: A Comparison of Steroidal Excretion and Metabolic Effects Induced in Man by Stress and by ACTH, Recent Progr. Hormone Res. **10**: 471, 1954.
32. Franksson, C., Gemzell, C. A., and von Euler, U. S.: Cortical and Medullary Adrenal Activity in Surgical and Allied Conditions, J. Clin. Endocrinol. **14**: 608, 1954.
33. Moore, F. D.: Endocrine Changes After Anesthesia, Surgery, and Unaesthetized Trauma in Man, Recent Progr. Hormone Res. **8**: 511, 1957.
34. Widström, G., and Claeson, K.: The Estimation of Iodine in Urine, Acta chem. scandinav. **13**: 606, 1959.
35. Berson, S. A.: Pathways of Iodine Metabolism. Am. J. Med. **20**: 653, 1956.
36. Spencer, W. A., Jackson, R. R., Vallbona, C., and Harrison, G. M.: Circulatory Disturbances in Life-Threatening Poliomyelitis, Am. Heart J. **59**: 384, 1960.
37. Remenchik, A. P., Dyniecwicz, J. M., and Schoenberger, J. A.: Changes in Body Composition During the Course of Acute Anterior Poliomyelitis, J. Lab. & Clin. Med. **53**: 195, 1959.
38. Ayres, P. J., Barlow, J., Garrod, O., Tait, S. A. S., Tait, J. F., and Walker, G.: Correlation of Adrenal Steroid Concentrations and Changes in Electrolyte Metabolism, Scandinav. J. Clin. & Lab. Invest. **10**: 29, 1957.
39. August, J. T., Nelson, D. H., and Thorn, G. W.: Response of Normal Subjects to Large Amounts of Aldosterone, J. Clin. Invest. **37**: 1549, 1958.
40. Hökfelt, B., and Skanze, B.: Effect of Aldosterone in a Case of Hypoaldosteronism, Acta endocrinol. **33**: 511, 1960.
41. Bartter, F. C., Liddle, G. W., Duncan, L. E., Jr., Barber, J. K., and Delea, C.: The Regulation of Aldosterone Secretion in Man: the Role of Fluid Volume, J. Clin. Invest. **35**: 1306, 1956.
42. Farrell, G.: Regulation of Aldosterone Secretion, Physiol. Rev. **38**: 709, 1958.
43. Muller, A. F., Riondel, A. M., and Manning, E. L.: Effect of Corticotrophin on Secretion of Aldosterone, Lancet **2**: 1021, 1956.
44. Luft, R., and von Euler, U. S.: Excretion of Catechol Amines During Administration of ACTH, Cortisone and Desoxy-Corticosterone Acetate, Metabolism **1**: 179, 1952.

Ultrafiltration studies on serum magnesium in normal and diseased states

ANANDA S. PRASAD, M.B., B.S., PH.D.,*
EDMUND B. FLINK, M.D., PH.D., and
ROBERT McCOLLISTER, M.D. *Minneapolis, Minn.*

Magnesium, being partly bound to proteins, exists in human serum as ultrafiltrable and nonultrafiltrable forms. Ultrafiltrable magnesium was determined in approximately 140 normal and diseased subjects.

Mean total and ultrafiltrable magnesium in normal sera were 1.809 ± 0.132 mEq. per liter and 1.175 ± 0.096 mEq. per liter, respectively. Increased ultrafiltrable magnesium was found in multiple myeloma, uremia, hypothyroidism, disseminated lupus erythematosus, hyperparathyroidism, generalized peritonitis, and myasthenia gravis. Increased ultrafiltrable magnesium always was associated with increased total magnesium. Decreased level was found in nephrotic syndrome, hyperthyroidism, alcoholic liver cirrhosis, delirium tremens, disseminated lupus erythematosus, and vitamin D–resistant rickets. Some patients had normal total but decreased ultrafiltrable magnesium.

Serum proteins, thyroid, and renal functions are some of the factors responsible for maintenance of normal level of total and ultrafiltrable magnesium. However, some other unknown factor or factors also seem to be operating for maintenance of normal magnesium level.

Increased percentage of ultrafiltrable magnesium usually was associated with decreased levels of protein fractions which bind magnesium. Decreased percentage was found in uremia, hypothyroidism, alcoholic liver cirrhosis, multiple myeloma, nephrotic syndrome, and hyperthyroidism. Possible reasons for these findings are discussed.

Magnesium has an important physiologic role in many enzymatic reactions.[1] Recently, clinical syndrome resulting from magnesium deficiency has been emphasized.[2] Being a divalent ion and being partly bound to proteins, it exists in

From the Departments of Medicine, University Hospital and Veterans Administration Hospital, Minneapolis, Minn.

Presented at the Sixth International Congress of Internal Medicine, on August 26, 1960, at Basel, Switzerland.

Received for publication April 28, 1960.

*Present address: Assistant Professor of Medicine, in Nutrition, Vanderbilt University, c/o Naval American Research Unit-3, Cairo, U. A. R.

Table I. Patients with increased ultrafiltrable magnesium (greater than 1.4 mEq. per

	Magnesium in mEq. per liter					
						Proteins in
Patient No.	*Total*	*Ultrafiltrable*	*% Ultrafiltrable magnesium*	*Total*	*Albumin*	*Alpha$_1$*
1	2.16	1.43	66.2	8.3	2.30	0.30
2	2.82	1.73	61.3	5.9	2.13	0.48
3	2.64	1.77	67.0	3.8	1.00	0.20
4	2.48	1.68	67.7	8.2	2.30	0.50
5	2.32	1.43	61.6	5.5	2.23	0.48
6	2.64	1.66	63.0	—	—	—
7	2.32	1.53	65.9	—	—	—
8	3.04	2.11	64.4	5.5	2.28	0.50
9	3.20	2.02	63.1	—	—	—
10	—	1.96	—	6.5	3.00	—

human serum as ultrafiltrable and nonultrafiltrable forms. Controversial findings regarding ultrafiltrable magnesium have been reported in patients with thyroid diseases.[3-6] Silverman and Gardner[7] reported the results of ultrafiltrable magnesium determination in a few cases of nephrosis, chronic nephritis, hypoparathyroidism, and in 2 cases of vitamin D intoxication. Since the ultrafiltrable magnesium in the serum represents the extracellular fluid concentration of this ion, it was considered important that this be investigated in a large series of normal and diseased states. We investigated the status of ultrafiltrable magnesium in approximately 140 subjects, the results of which will be presented.

Methods

Magnesium was determined by the method of Garner.[8] Ultrafiltration was done by a technique reported previously.[9] Total protein was determined by the biuret method.[10] Paper electrophoresis was used for the determination of different protein fractions.[11] Total magnesium was determined in serum samples.

Results

In our laboratory the mean for 16 magnesium determinations of the same serum sample was 1.78 ± 0.08 mEq. per liter. The result of recovery experiment when 1 mEq. of magnesium was added to normal saline was 1.02 ± .05 mEq. In series of recovery experiments the error falls within the range usually expected in colorimetric determinations.[8]

In 36 normal subjects the mean total magnesium was 1.809 (S.D. ± 0.132) mEq. per liter and the mean ultrafiltrable magnesium was 1.175 (S.D. ± 0.098) mEq. per liter. The mean percentage of ultrafiltrable magnesium was 64.0 (S.D. ± 2.4). The values beyond 2 standard deviations from the normal mean were considered abnormal.

liter)

gram per cent			
Alpha$_2$	*Beta*	*Gamma*	*Diagnosis*
0.70	0.70	4.30	Multiple myeloma
1.16	1.01	1.12	Multiple myeloma
1.00	0.80	0.80	Uremia
2.36	2.05	1.00	Uremia
0.72	1.15	0.89	Uremia
—	—	—	Cretin
—	—	—	Hyperparathyroidism
0.50	0.68	1.54	Disseminated lupus erythematosus BUN-117 mg. %
—	—	—	Generalized peritonitis
—	—	—	Myasthenia gravis

Fig. 1 shows the results in 10 patients with multiple myeloma. The total magnesium was increased in 4 and decreased in 2 cases. The ultrafiltrable magnesium was normal in all except 2 where it was increased.

Results obtained in renal diseases also are shown in Fig. 1. Eight cases of nephrosis were investigated and in each the total and ultrafiltrable magnesium were decreased in 2 instances. The percentage of ultrafiltrable magnesium was increased in 3 cases associated with hypoalbuminemia. Of 11 patients with uremia, 5 had increased total and 2 had increased ultrafiltrable magnesium.

In 3 patients with hyperthyroidism the total magnesium and in 4 the ultrafiltrable magnesium was decreased (Fig. 1). In hypothyroidism increased total magnesium was found in 3 cases. The ultrafiltrable magnesium was increased in 1 case only and the percentage of ultrafiltrable magnesium was decreased in 3 cases. One patient with hyperparathyroidism had increased total and ultrafiltrable magnesium, and one patient with hypoparathyroidism had normal values. The latter was receiving vitamin D and calcium therapy. The percentages for ultrafiltrable magnesium were normal in both.

In 20 cases of cirrhosis the total magnesium in 6 and the ultrafiltrable magnesium in 5 were decreased (Fig. 1). In 14 cases where protein electrophoretic patterns were available, the total serum magnesium correlated well with the level of serum albumin ($r = 0.7$, S.E. $= 0.27$).

Results in collagen diseases are also presented in Fig. 1. Seven cases of rheumatoid arthritis had normal total and ultrafiltrable magnesium. One with hypoalbuminemia had low total but normal ultrafiltrable magnesium. Four patients had increased percentage of ultrafiltrable magnesium. One out of 4 cases with systemic disseminated lupus erythematosus had decreased and 1 had increased total and ultrafiltrable magnesium. An increased percentage of ultrafiltrable fraction was found in 3 instances. One patient with scleroderma had normal values.

In addition, 1 patient with arrhenoblastoma, 4 with emphysema, 2 with hypogammaglobulinemia, 2 with macroglobulinemia, 1 with carcinoma of the lung, 2 with leukemic reticuloendotheliosis, and 2 with ulcerative colitis had normal total and ultrafiltrable magnesium. One patient had generalized peritonitis and the total and ultrafiltrable magnesium were 3.20 and 2.02 mEq. per liter, respectively. One 16-month-old boy had vitamin D–resistant rickets. His total and ultrafiltrable magnesium were 1.52 and 0.88 mEq. per liter before therapy and, after 3 weeks of treatment with vitamin D, the total and ultrafiltrable magnesium were 1.93 and 1.23 mEq. per liter. One patient with myasthenia gravis with normal proteins had 1.96 mEq. per liter ultrafiltrable magnesium. Three adults with muscular dystrophy had total magnesium ranging from 2.08 to 2.12 mEq. per liter and ultrafiltrable magnesium was 1.32 mEq. per liter in 1 case in which it was determined. Four patients with delirium tremens had decreased total magnesium and 1 case had decreased ultrafiltrable magnesium.

Discussion

Hopkins and associates[12] found 83 per cent of magnesium as ultrafiltrable in normal subjects. Silverman and Gardner,[7] using the same technique as Hopkins and associates,[12] obtained the following values in normal subjects: Total magnesium 1.77 ± .02 mEq. per liter; ultrafiltrable magnesium, 1.16 ± 0.02 mEq. per liter; and percentage ultrafiltrable, 65.3 ± 1.8 of total magnesium. Our results in normal subjects are in close agreement with those of Silverman and Gardner.[7] These authors used the modified ultrafiltration techniques described by Lavietes.[13]

Table II. Patients with decreased ultrafiltrable magnesium (less than 0.99 mEq. per

	Magnesium in mEq. per liter					
						Proteins in
Patient No.	*Total*	*Ultrafiltrable*	*% Ultrafiltrable magnesium*	*Total*	*Albumin*	*Alpha$_1$*
1	1.66	0.95	57	4.60	1.80	—
2	1.50	0.93	62	3.80	1.23	0.34
3	1.50	0.97	65	7.50	3.36	0.46
4	1.36	0.90	66	5.90	3.24	0.42
5	1.52	0.93	61	—	—	—
6	1.76	0.92	52	6.79	3.07	0.43
7	1.23	0.85	69	6.30	1.49	0.34
8	1.30	0.83	64	6.04	2.50	0.34
9	1.34	0.83	62	7.00	1.87	0.39
10	1.68	0.84	50	7.90	2.70	—
11	1.70	0.90	54	—	—	—
12	1.45	0.96	66	—	—	—
13	1.36	0.95	70	7.70	2.20	0.42
14	1.52	0.88	58	—	—	—

In some cases of multiple myeloma the total serum and ultrafiltrable magnesium were increased but the percentage of ultrafiltrable magnesium was normal. Two patients had low total but normal ultrafiltrable magnesium. Both of these had hypoalbuminemia, and in one, alpha$_1$ and alpha$_2$ globulins were also decreased, all of which bind magnesium.[14] The increased total magnesium could be due to release of magnesium from skeleton and/or renal dysfunction, which often accompanies this disease.

In nephrotic syndrome, the total magnesium was somewhat lower than normal, probably because of hypoproteinemia. The percentage of ultrafiltrable magnesium was increased in 3 cases associated with hypoalbuminemia. Some patients with uremia had increased total and ultrafiltrable magnesium; however, the percentage of ultrafiltrable magnesium remained normal in the majority. Similar results in nephrosis in children and in chronic glomerular nephritis have been reported by Silverman and Gardner.[7]

According to Soffer and co-workers,[6] the protein-bound magnesium was increased in hyperthyroidism and no bound magnesium was present in myxedema. This was later confirmed by Dine and Lavietes.[5] However, Cope and Wolff[4] found no difference in cases of hyperthyroidism from the normal subjects. Silverman and Gardner[7] investigated 5 cretin and 2 juvenile hypothyroid patients and found no abnormality with respect to magnesium.

Our results indicate that in hyperthyroidism the magnesium level in serum and its ultrafiltrate tends to be lower than normal. This is consistent with the findings of Tapley[15] who obtained magnesium diuresis following administration of L-triiodothyronine to myxedematous patients. In some hypothyroid patients in our series the total and ultrafiltrable magnesium were higher than normal. Some

liter)

gram per cent			
Alpha$_2$	*Beta*	*Gamma*	*Diagnosis*
—	—	—	Nephrotic syndrome
0.85	0.19	1.19	Nephrotic syndrome
1.05	0.92	1.72	Hyperthyroidism
0.57	0.91	0.76	Hyperthyroidism
—	—	—	Hyperthyroidism
0.76	1.23	1.30	Hyperthyroidism
0.30	0.72	3.45	Cirrhosis
0.81	0.54	1.85	Cirrhosis
0.59	0.79	3.36	Cirrhosis
—	—	—	Cirrhosis
—	—	—	Cirrhosis
—	—	—	Delirium tremens
0.65	1.00	3.46	Disseminated lupus erythematosus
—	—	—	Vitamin D–resistant rickets before therapy

Table III. Patients with increased percentage of ultrafiltrable magnesium (greater than 69%)

Patient No.	Magnesium in mEq. per liter			Proteins in gram per cent						Diagnosis
	Total	Ultrafiltrable	% Ultrafiltrable magnesium	Total	Albumin	$Alpha_1$	$Alpha_2$	Beta	Gamma	
1	1.44	1.05	72.9	6.5	2.44	0.57	0.98	0.73	1.79	Multiple myeloma
2	1.51	1.15	76.2	7.1	2.60	0.20	0.60	3.30	0.40	Multiple myeloma
3	1.66	1.28	77.1	5.6	1.60	—	—	—	—	Nephrotic syndrome
4	1.54	1.13	73.4	3.0	0.60	—	—	—	—	Nephrotic syndrome
5	1.44	1.00	69.4	3.7	0.60	0.20	1.30	1.10	0.50	Nephrotic syndrome
6	1.23	0.85	69.1	6.3	1.49	0.34	0.30	0.72	3.45	Hemachromatosis
7	1.48	1.04	70.3	6.9	3.04	0.47	0.41	0.70	2.28	Cirrhosis
8	1.74	1.23	70.7	5.5	1.86	0.31	0.74	0.74	1.86	Cirrhosis
9	1.97	1.37	69.5	7.4	1.94	0.42	0.93	0.98	3.12	Cirrhosis
10	1.44	1.10	76.4	7.3	1.70	0.33	0.39	1.17	3.71	Cirrhosis
11	1.74	1.21	69.5	5.5	2.10	0.49	0.71	0.79	1.40	Hepatitis
12	1.50	1.25	83.0	—	—	—	—	—	—	Delirium tremens
13	1.50	1.25	83.0	—	—	—	—	—	—	Delirium tremens
14	1.88	1.40	74.5	—	—	—	—	—	—	Delirium tremens
15	1.86	1.30	70.0	—	—	—	—	—	—	Delirium tremens
16	1.48	1.20	81.1	—	—	—	—	—	—	Delirium tremens
17	1.50	1.14	76.0	7.9	1.00	0.50	1.21	1.75	3.45	Rheumatoid arthritis
18	1.80	1.27	70.6	5.6	2.72	0.35	0.62	0.66	2.12	Rheumatoid arthritis
19	1.66	1.18	71.0	8.1	2.90	—	—	—	—	Rheumatoid arthritis
20	1.74	1.26	72.4	5.9	2.60	0.36	0.71	1.03	1.19	Rheumatoid arthritis
21	3.04	2.11	69.4	5.5	2.28	0.50	0.50	0.68	1.54	Disseminated lupus erythematosus

authors have observed a great similarity between symptoms of magnesium deficiency and thyrotoxicosis and between myxedema and known effects of magnesium excess.[15] Hueber[16] has reported clinical improvements in patients with thyrotoxicosis following parentral administration of magnesium.

Harmon[17] has reported magnesium deficiency in a child with parathyroid adenoma. Injection of parathyroid extract to normal dogs resulted in increased urinary excretion of magnesium and a delayed fall in urinary excretion, followed by a rise in serum magnesium, the latter effect probably being due to renal damage produced by the parathyroid extract.[18] Parathyroidectomy in normal dogs produced little effect on magnesium balance except a minor decrease in serum level.[18] Watchorn and McCance[19] observed a decrease in total and ultrafiltrable magnesium following parathyroid extract administration. Silverman and Gardner[7] have found decreased total and ultrafiltrable magnesium in 3 patients with hypoparathyroidism. In our study (Fig. 1), in 1 patient with

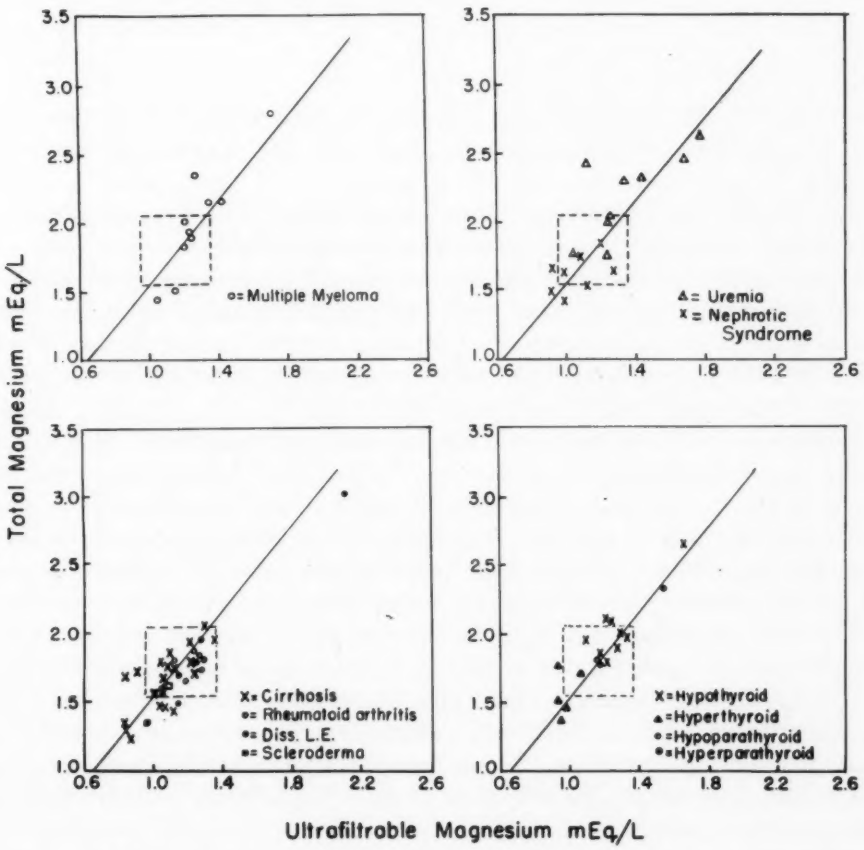


Fig. 1. Central rectangular area has been drawn to represent normal mean values for total and ultrafiltrable magnesium with a standard deviation range of 2. The straight line has been drawn assuming the normal mean percentage of ultrafiltrable magnesium to be 64 per cent.

Table IV. Patients with decreased percentage of ultrafiltrable magnesium (less than

	Magnesium in mEq. per liter					
						Proteins in
Patient No.	*Total*	*Ultrafiltrable*	*% Ultrafiltrable magnesium*	*Total*	*Albumin*	*Alpha$_1$*
1	2.34	1.29	55.1	5.7	3.20	0.43
2	1.66	0.95	57.2	4.6	1.80	—
3	2.30	1.33	57.8	5.3	2.81	0.28
4	2.44	1.12	45.9	5.5	2.90	0.31
5	1.78	1.04	58.5	5.6	2.60	—
6	1.76	0.92	52.3	6.8	3.07	0.43
7	2.08	1.22	58.6	7.0	3.06	0.34
8	1.94	1.08	55.8	—	—	—
9	2.14	1.20	56.0	6.0	2.88	—
10	1.68	0.84	50.0	7.9	2.70	—
11	1.70	0.90	54.1	—	—	—

hyperparathyroidism the total and ultrafiltrable magnesium were increased and in a hypoparathyroidism patient on treatment, the magnesium levels were normal.

In alcoholic cirrhosis of the liver, the decreased total magnesium is most likely due to associated hypoalbuminemia. This is supported by the fact that a significant positive correlation was observed between the total magnesium and the serum albumin concentration in the cases investigated. Low serum magnesium in these cases has been ascribed by some authors to low dietary intake.[20] Excess urinary excretion of magnesium in alcoholism has been noted and this may be another contributing factor in such patients.[21]

Silverman and Gardner[7] found decreased serum magnesium in 2 cases of vitamin D intoxication, whereas Kruger[22] reported a decrease in serum magnesium in vitamin D deficiency cases. In rats magnesium absorption increases slightly with vitamin D and addition of calcium carbonate, sodium bicarbonate, or inorganic phosphate did not affect its absorption; however, addition of phytic acid reduced the absorption somewhat.[23] Our results in one child with vitamin D–resistant rickets showed a definite increase in serum magnesium following adequate vitamin D therapy. Although this situation is not comparable to the one reported by Silverman and Gardner,[7] the results are nonetheless interesting.

Obviously abnormal ultrafiltrable magnesium in presence of normal total magnesium and vice versa can exist in human serum under different conditions.

Magnesium is mainly an intracellular ion. The level of ultrafiltrable magnesium in serum which can be regarded as representing the extracellular fluid concentration appears to remain constant in normal subjects within reasonable limits. The mechanism which governs this equilibrium between the intra- and extracellular magnesium concentration is not well understood. In the serum, the law of mass action determines the magnesium protein relationship,[14] and as such

59 per cent)

gram per cent			
Alpha$_2$	*Beta*	*Gamma*	*Diagnosis*
0.70	0.60	0.80	Multiple myeloma
—	—	—	Nephrotic syndrome
0.53	0.57	1.11	Uremia
0.59	0.69	1.05	Uremia
—	—	—	Uremia
0.76	1.23	1.30	Hyperthyroid
0.85	1.16	1.62	Hypothyroid
—	—	—	Hypothyroid
—	—	—	Hypothyroid
—	—	—	Cirrhosis
—	—	—	Cirrhosis

the proteins affect the percentage of ultrafiltrable magnesium. The status of renal function also determines the serum level of magnesium.[24-26]

Several factors seem to be responsible for maintenance of magnesium level in the serum and the ultrafiltrate. Serum proteins, thyroid, and renal functions are important factors in the presence of normal dietary intake. However, apparently in addition to the above-mentioned factors, some other unknown factor or factors must be operating to maintain the level of ultrafiltrable magnesium within a narrow range.

Increased ultrafiltrable magnesium was found in 2 patients with multiple myeloma, 3 with uremia, and one each with hypothyroidism, disseminated lupus erythematosus, hyperparathyroidism, generalized peritonitis, and myasthenis gravis (Table I). Increased level of ultrafiltrable magnesium was always associated with increased total magnesium. Recently, hypermagnesemia of renal disease has been attributed to the degree of reduction in glomerular filtration rate.[24] This would tend to increase the ultrafiltrable magnesium first. In multiple myeloma and disseminated lupus erythematosus renal lesions develop and as such one may expect similar phenomena in those conditions. Since thyroid excess causes increased magnesium excretion,[15] one may postulate that hypothyroidism may be associated with decreased renal excretion of magnesium. Excess parathormone may cause increase in serum magnesium due to renal damage[18]; therefore, one may expect increased serum and ultrafiltrable magnesium in hyperparathyroidism. Thus the increased level of total and ultrafiltrable magnesium in cases of multiple myeloma, uremia, disseminated lupus erythematosus, hypothyroidism, and hyperparathyroidism may be on the basis of renal impairment alone. The mechanism responsible for the increase in ultrafiltrable magnesium in cases of generalized peritonitis and myasthenia gravis is not clear at present.

Decreased ultrafiltrable magnesium was found in 14 cases. This included 2 patients with nephrotic syndrome, 4 with hyperthyroidism, 5 with alcoholic cirrhosis of the liver, 1 with delirium tremens, 1 with disseminated lupus erythematosus, and 1 with vitamin D–resistant rickets (Table II). In 10, it was associated with decreased total magnesium. Four patients (1 with nephrotic syndrome, 1 with hyperthyroidism, and 2 with alcoholic cirrhosis of the liver) had normal total magnesium but decreased ultrafiltrable magnesium. In these cases the renal excretion of magnesium probably was increased, which would tend to decrease the ultrafiltrable magnesium initially. Later, since the magnesium-protein relationship obeys the law of mass action, the protein-bound magnesium would decrease, to establish a new equilibrium with respect to the ultrafiltrable fraction. If this process continues eventually a low total magnesium would be expected. There is evidence to suggest that excess renal magnesium excretion may be expected in hyperthyroidism[15] and in alcoholism.[21] In nephrotic syndrome also this may be the case because of renal tubular damage which might interfere with magnesium reabsorption. The decreased level of total and ultrafiltrable magnesium in 1 patient with disseminated lupus erythematosus could have been due to hypoalbuminemia. Whether or not renal factors played any role cannot be established from our observations. This explanation does presume that the equilibrium between ultrafiltrable and bound magnesium is being reached slowly. We have no evidence to prove that this is the case with respect to magnesium ion, and as such this should be regarded as mere speculation.

Increased percentage of ultrafiltrable magnesium was present in 21 instances (Table III). Serum electrophoretic pattern was not available in all cases, but in those where available, the increased percentage of ultrafiltrable magnesium was correlated well with hypoalbuminemia with one exception only. This is similar to the results obtained for ultrafiltrable calcium which show increased percentage in presence of hypoalbuminemia.[27] Although hypoalbuminemia affects the percentage of ultrafiltrable magnesium, the absolute level of magnesium in the ultrafiltrate is determined by other factors, as discussed previously.

Table IV presents 11 patients who had decreased percentage of ultrafiltrable magnesium. Four of these had increased total magnesium (1 multiple myeloma, 2 uremia, and 1 hypothyroidism) but normal ultrafiltrable magnesium and serum albumin was greater than 2.8 Gm. per cent in all those cases. The explanation for the presence of increased protein-bound magnesium under these conditions is not clear. Four patients had normal total but decreased absolute level and percentage of ultrafiltrable magnesium (1 nephrotic syndrome, 1 hyperthyroidism, and 2 cirrhosis of the liver). As discussed above, in these conditions there may be increased urinary loss of magnesium which thereby decreases the absolute level and percentage of ultrafiltrable magnesium. Three patients (1 with uremia and 2 with hypothyroidism) had normal total and ultrafiltrable magnesium, but the percentage of ultrafiltrable magnesium was decreased.

REFERENCES

1. White, A., Handler, P., Smith, E. L., and Stetten, Dew, Jr.: Principles of Biochemistry, New York 1954, McGraw-Hill Book Co., Inc.

2. Flink, E. B., McCollister, R., Prasad, A. S., Melby, J. C., and Doe, R. P.: Evidences for Clinical Magnesium Deficiency, Ann. Int. Med. **47**: 956, 1957.
3. Bissell, G. W.: Magnesium Partition in Hyperthyroidism With Special Reference to Effect of Thiouracil, Am. J. M. Sc. **210**: 195, 1945.
4. Cope, C. L., and Wolff, B.: The Ultrafiltrable Magnesium in Hypothyroidism, Biochem. J. **36**: 413, 1942.
5. Dine, R. F., and Lavietes, P. H.: Serum Magnesium in Thyroid Diseases, J. Clin. Invest. **21**: 781, 1942.
6. Soffer, L. J., Dantes, D. A., Grossman, E. B., Sobotka, H., and Jacobs, M. D.: Ultrafiltrable Magnesium in Hyperthyroidism, J. Clin. Invest. **18**: 597, 1939.
7. Silverman, S. H., and Gardner, L. I.: Ultrafiltration Studies on Serum Magnesium, New England J. Med. **250**: 938, 1954.
8. Garner, R. J.: Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow, Biochem. J. **40**: 828, 1946.
9. Prasad, A. S., and Flink, E. B.: Effect of Carbon Dioxide on Concentration of Calcium in an Ultrafiltrate of Serum Obtained by Centrifugation, J. Appl. Physiol. **10**: 103, 1957.
10. Weichselbaum, J. E.: An Accurate and Rapid Method for the Determination of Proteins in Small Amounts of Blood Serum and Plasma, Am. J. Clin. Path. **16**: 40, 1946.
11. Kunkel, H. G., and Tiselius, A.: Electrophoresis of Proteins on Filter Paper, J. Gen. Physiol. **35**: 89, 1951.
12. Hopkins, T. R., Howard, J. E., and Eisenberg, H.: Ultrafiltration Studies on Calcium and Phosphorus in Human Serum, Bull. Johns Hopkins Hosp. **91**: 1, 1952.
13. Lavietes, P. H.: Anaerobic Ultrafiltration, J. Biol. Chem. **120**: 267, 1937.
14. Prasad, A. S., Flink, E. B., and Zinneman, H. H.: The Base Binding Property of the Serum Proteins With Respect to Magnesium, J. LAB. & CLIN. MED. **54**: 357, 1959.
15. Tapley, D. F.: Magnesium Balance in Myxedematous Patients Treated With Tri-iodothyronine, Bull. Johns Hopkins Hosp. **96**: 274, 1955.
16. Von Hueber, E. F.: Ueber die Beeinflussung von Hyperthyreosen durch Magnesiumglutaminat, Wien. klin. Wchnschr. **52**: 932, 1939.
17. Harmon, M.: Parathyroid Adenoma in a Child, A. M. A. J. Dis. Child. **91**: 313, 1956.
18. Roberts, B., Murphy, J. J., Miller, L., and Rosenthal, O.: The Effect of Parathyroid Hormone Upon Serum Levels and Urinary Excretion of Magnesium, Surg. Forum **5**: 509, 1954-55.
19. Watchorn, E., and McCance, R. A.: Inorganic Constituents of CSF-II. The Ultrafiltration of Calcium and Magnesium From Human Sera, Biochem. J. **26**: 54, 1932.
20. Stutzman, F. L., and Amatuzio, D. S.: Blood Serum Magnesium in Portal Cirrhosis and Diabetes Mellitus, J. LAB. & CLIN. MED. **41**: 215, 1953.
21. McCollister, R., Prasad, A. S., Doe, R. P., and Flink, E. B.: Normal Renal Magnesium Clearance and the Effect of Water Loading, Chlorothiazide, and Ethanol on Magnesium Excretion, J. LAB. & CLIN. MED. **52**: 928, 1958. (Abst.)
22. Kruger, E.: Klinisch-experimentelle Untersuchungen über die Veranderungen des magnesiumgehaltes in verschiedenen Altersstufen, insbesondere bei Rachitis und Tetanie der Kinder, Ztschr. F. Kinderh. **53**: 83, 1932.
23. Meintzer, R. B., and Steenbock, H.: Vitamin D and Magnesium Absorption, J. Nutrition **56**: 285, 1955.
24. Robinson, R. R., Murdaugh, H. V., and Peschel, E.: Renal Factors Responsible for the Hypermagnesemia of Renal Diseases, J. LAB. & CLIN. MED. **53**: 572, 1959.
25. Hammarsten, J. F., and Smith, W. D.; Serum Magnesium in Renal Diseases, Clin. Res. **6**: 152, 1958.
26. Hirschfelder, A. D.: Effect of Renal Insufficiency Upon Plasma Magnesium and Magnesium Excretion After Ingestion of Magnesium Sulfate, J. Biol. Chem. **104**: 647, 1934.
27. Prasad, A. S., and Flink, E. B.: The Determination of Ultrafiltrable Calcium in a Variety of Clinical Conditions, J. LAB. & CLIN. MED. **52**: 1, 1958.

Changes in serum and spinal fluid calcium effected by disodium ethylenediaminetetraacetate

ALFRED SOFFER, M.D., and TAFT TORIBARA, PH.D. *Rochester, N. Y.*

This study was done to determine the qualitative and quantitative changes in serum and spinal fluid calcium effected by the chelating agent, disodium ethylenediaminetetraacetate. Wet ashing and determination of ultrafiltrable calcium before and after drug administration demonstrated that there was no significant change in total calcium. A marked fall in protein-bound and a definite increase in ultrafiltrable calcium reflected a major decrease in the ionic calcium. The average decrease in the ionic fraction was 1.5 mg. per 100 ml. Total spinal fluid calcium showed no change. This provides evidence that disodium edathamil does not cross the blood-spinal fluid barrier in significant quantities during the average therapy period.

Shortly after the initiation of clinical studies of induced hypocalcemia[1] it became apparent that there was little or no knowledge concerning the magnitude of ionic calcium changes effected by the utilization of chelating agents. It was decided, therefore, to determine ultrafiltrable and total ashed calcium before and after intravenous administration of varying amounts of disodium ethylenediaminetetraacetate (disodium EDTA or edathamil). In order to evaluate the movement of this compound across the blood-spinal fluid barrier, simultaneous serum and spinal fluid samples were obtained before and after therapy.

With increasing use of EDTA in medicine there is the need for an evaluation of the analytic conditions necessary to determine EDTA-bound calcium in a biologic sample. In the presence of a chelating agent determination of serum calcium by standard procedures may give misleading results if the exact pH is not recognized as a critical factor. A simple and accurate method was devised for the determination of calcium in solutions containing EDTA.

From the Department of Radiation Biology, University of Rochester, Schools of Medicine and Dentistry, and the Cardiopulmonary Laboratories of the Rochester General Hospital, Rochester, N. Y.

Based in part on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

Received for publication Jan. 31, 1961.

Materials and methods

Nine patients with a mean age of 56 years were studied. The clinical status of these subjects is described in Table I. All subjects were normocalcemic. Ethylenediaminetetraacetic acid (edathamil) was supplied as the disodium salt (Endrate) by Dr. George Berryman of Abbott Laboratories. This drug was given intravenously in dosages of from 0.5 to 4.0 Gm. in periods ranging from ½ hour to 11 hours. The drug was diluted in either 500 or 1,000 ml. of 5 per cent glucose in water. In 3 instances 3 Gm. of the drug was administered in ½ hour. In all other cases the maximum rate of infusion was 2 Gm. in 30 minutes. For determining total calcium it was found best to use a trichloroacetic acid (TCA) deproteinized sample. Two milliliters of serum were placed in a 10 ml. volumetric flask and 5 ml. of 10 per cent TCA was diluted to volume with distilled water. The entire sample was transferred to a 15 ml. conical centrifuge tube and the protein precipitate was separated by centrifugation. Five milliliters of the supernatant liquid were used for the calcium determination by flame photometry. Because the samples in the present experiment contained some EDTA, it first was necessary to determine whether the precipitation of the calcium with oxalate would be complete. When equivalent quantities of calcium and EDTA were treated with an excess of oxalate a recovery of 84 per cent was obtained. When the samples of calcium and EDTA were first evaporated to dryness and then heated at a boiling temperature with 1 ml. of concentrated nitric acid until most of the acid had been volatized, the same recoveries of calcium were obtained as in the case when no EDTA was present. Therefore, all samples for this experiment were evaporated to dryness and given the nitric acid treatment prior to precipitation of the calcium as the oxalate. From 3 patients 1 ml. samples of spinal fluid were taken and the calcium, as the oxalate, was separated by precipitation according to standardized procedures. The calcium oxalate was dissolved in dilute hydrochloric acid made up to volume and determined in a flame photometer. Ultrafiltrable serum calciums were determined in 6 patients by the method of Toribara and co-workers.[2] Four milliliters of fresh serum were placed in a cellophane bag, which then was placed in the Toribara ultrafiltration apparatus; the apparatus was flushed for 3 minutes with a 5 per cent CO_2 – 95 per cent O_2 mixture and then closed. The apparatus was placed in a centrifuge and spun at 2,000 r.p.m. until a sample of 1 ml. of ultrafiltrate could be taken (about 4 hours). Old serum, even when kept in a frozen state, undergoes changes which make it impossible to obtain uniform samples. It is therefore of the utmost importance to separate the serum and measure aliquots as soon as possible. For the reaction $Ca + Pr \rightleftharpoons CaPr$, McLean and Hastings[3] have calculated the constant $K = \frac{CaPr}{(Ca^{++})\ (Pr)} = 166$. In normal serum the ionic calcium was determined by the same authors to be about 1.3 by 10^{-3} molar. Substituting this value in the above equation, the ratio of unbound protein to calcium-bound protein equals approximately 5. If we make the reasonable assumption that the total calcium-binding sites on protein remain the same

Table I. Clinical status of subjects

Subject	*Sex*	*Age in years*	*Clinical status*
A	F	86	ASHD*
B	F	71	ASHD with cardiomegaly
C	F	82	ASHD with cardiomegaly
D	M	59	ASHD with cardiomegaly
E	F	43	Normal
F	F	42	Progressive systemic scleroderma
R. M.	M	38	RHD† with aortic stenosis
B. S.	F	16	Progressive systemic scleroderma
V. K.	F	46	Polyarteritis nodosa with myocarditis

*ASHD signifies atherosclerotic heart disease.
†RHD signifies rheumatic heart disease.

Table II. Effects of disodium EDTA on calcium content corrected to original serum volume (calcium as milligram per 100 ml.)

Subject	*Dosage of Na_2 EDTA*		*Total calcium*	*Ultra-filtrable calcium*	*Calcium proteinate*	*Ionic calcium*
A	3.7 Gm.	Before	8.2	4.60	3.60	3.4
	in 35 minutes	After	8.9	6.80	2.10	1.9
B	4 Gm.	Before	9.4	5.61	3.79	4.4
	in 1 hour	After	9.0	6.53	2.47	2.7
C	3 Gm.	Before	8.2	3.91	4.29	2.7
	in 1½ hours	After	8.3	6.10	2.20	1.3

before and after EDTA infusion, we can express the ionic calcium levels by the expression $Ca^{++} = \frac{CaPr}{K\ (Pr)}$.

Results

The data in Table II demonstrate that disodium EDTA produces a dramatic decrease in serum ionic calcium. In the 3 experiments listed the average drop in serum ionic calcium was 1.5 mg. per 100 ml. Table III lists the results of 7 experiments in 6 subjects. In 4 instances the simultaneous serum and spinal fluid calcium changes are compared (Cases A, B, and C also are noted in Table II). In Table II the ultrafiltrable calcium values are corrected for filtrable water. The ultrafiltrate consists of 99 Gm. of water per 100 ml. Normal serum consists of 93 Gm. of water per 100 ml., of which 90 Gm. is filtrable. Since the spinal fluid calcium is entirely diffusible only the total spinal fluid calcium was determined. Disodium EDTA effected no significant change in spinal fluid calcium in spite of dramatic and simultaneous changes in serum ultrafiltrable calcium.

In a study described elsewhere[4] it was determined that calcium can be precipitated completely by oxalate in the presence of EDTA if the pH is kept below but near 5.0. If the pH is elevated to 8.0 during precipitation, any EDTA present will chelate completely an equimolar quantity of calcium. By precipitating calcium at a pH below 5.0 in one and above 8.0 in another aliquot of

Table III. Effects of disodium EDTA on calcium content of serum and spinal fluid (calcium content as milligram per 100 ml.)

Calcium	*Subject*						
	A	*B*	*C*	*D*	*E*	F_a	F_b*
TC_1	8.20	9.40	8.2		9.67	8.5	8.1
TC_2	8.90	9.00	8.3			7.2	7.4
UF_1	5.05	6.17	4.3	5.64	5.02	4.4	4.9
UF_2	7.50	7.18	6.7	6.56	5.62	4.3	
SpF_1	4.75		4.8	5.48	4.20		
SpF_2	4.43		4.3	5.14	4.30		

TC = total serum calcium, UF = untrafiltrable serum calcium, SpF = spinal fluid calcium. Subscript 1 refers to samples before EDTA administration; subscript 2 refers to samples after EDTA administration.

*Subject F was studied twice within a 30 day period.

Table IV. Amount of serum calcium complexed to disodium EDTA at termination of varying rates of infusion

Subject	*Duration of infusion (hours)*	*Disodium EDTA (grams)*	*Serum calcium pH 5.0 (mg. 100 ml.)*	*Serum calcium pH 8.0 (mg. 100 ml.)*	*Calcium complexed to Na_2 EDTA (mg. 100 ml.)*
R. M.	1	3.0	10.7	8.2	2.5
	2	3.0	9.5	8.0	1.5
	3	3.0	10.0	8.3	1.7
B. S.	7½	2.1	10.4	9.7	0.7
	11	2.5	10.2	9.2	1.0
V. K.	2	3.1	9.2	7.0	2.2

serum or plasma, the quantity of calcium complexed to EDTA in blood may be determined by difference. In order to obtain the necessary pH control an acetate buffer system was used at the lower and an ammonium system at the higher pH. This method, which is applicable in any clinical laboratory, was used to study the status of serum calcium following EDTA administration (Table IV). A significant amount of serum calcium was complexed to EDTA following even extremely slow rates of infusion (Subject B. S.).

Discussion

In normal serum approximately 60 per cent of the calcium is present in a diffusible ultrafiltrable form and a majority of this fraction is present as ionic calcium.[2, 5] A small amount is present as calcium complexed by such small anions as citrate, phosphate, and bicarbonate. Protein-bound calcium, which is nondiffusible and nonultrafiltrable, comprises approximately 40 per cent of total serum calcium. Sodium salts of EDTA bind serum calcium to form a stable, soluble, nonionized complex which is relatively rapidly excreted in the urine.

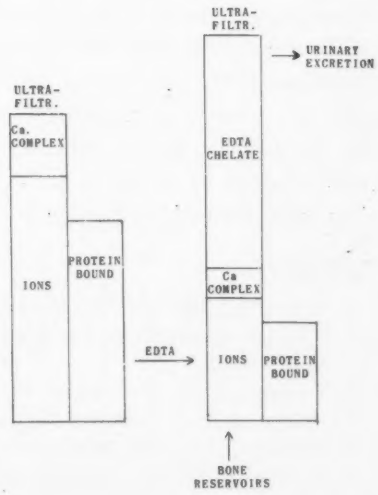


Fig. 1. Changes in fractions of serum calcium effected by administration of disodium EDTA. Total serum calcium remains unchanged.

This study demonstrates that EDTA acts predominantly upon the ionic fraction of serum calcium. The graphs in Fig. 1 demonstrate that disodium EDTA does not change total serum calcium but rather that chelation rearranges the distribution of calcium in the blood so that as the calcium chelate fraction rises, protein-bound and ionic calcium drop markedly. Urinary losses of calcium are replaced from bone reservoirs. Spencer and co-authors[6-8] have recorded a rise in total serum calcium following extremely rapid infusion of disodium edathamil in normocalcemic patients. We occasionally have administered this drug at comparable rates, but we have never been able to demonstrate a significant rise in total serum calcium. There was, of course, a difference in techniques, since Spencer utilized oxalate precipitation, but the total serum calcium in our studies was determined by flame photometry. One subject, a 42-year-old normocalcemic woman with progressive systemic scleroderma, manifested a fall in total serum calcium following chelation. The same response occurred on a subsequent trial 1 month later (Subject F, Table III). Spencer and associates[6] have recorded a fall in total serum calcium effected by disodium EDTA administration in a hypercalcemic patient.

Foreman[9] found that 1 hour after intravenous administration of C^{14}-labeled calcium EDTA the spinal fluid concentration was one-fortieth that of plasma. Using the disodium salt of EDTA our experiments show that this salt also is denied entrance into spinal fluid during the average therapeutic period.

The confusion that presently exists in the interpretation of serum calcium analyses following chelation is illustrated by the communication of Carr and Frank,[10] who maintain that following treatment with EDTA the true picture of calcium in serum can be obtained only if the serum pH is lowered to between 3 and 5. Under these conditions one will break completely the chelating bond and obtain, after oxalate precipitation, the total amount of calcium present in serum, but the observer will have no idea of the calcium changes effected by the chelating drug. Insight into calcium homeostasis may be achieved only if the exact amount of total calcium bound to EDTA is determined. This value may be obtained readily by difference if serum calcium is precipitated at pH values of 5 and 8 following EDTA administration. Only one sample of plasma need be obtained if this method is used. The clinician may wish to utilize the physiologic pH for a more accurate reflection of in vivo serum calcium changes. At a pH of 7.4 any EDTA present will be complexed to the extent of 97.5 per cent in the presence of excess oxalate. Therefore, a pH of either 8 or 7.4 is satisfactory for most clinical purposes.

REFERENCES

1. Soffer, A., Toribara, T., Moore-Jones, D., and Weber, D.: Clinical Indications and Untoward Reactions of Chelation in Cardiac Arrhythmias, A. M. A. Arch. Int. Med. **106**: 824, 1960.
2. Toribara, T. Y., Terepka, A. R., and Dewey, P. A.: The Ultrafiltrable Calcium of Human Serum. I. Ultrafiltration Methods and Normal Values, J. Clin. Invest. **36**: 738, 1957.
3. McLean, F. C., and Hastings, A. B.: The State of Calcium in the Fluids of the Body. I. The Conditions Affecting the Ionization of Calcium, J. Biol. Chem. **108**: 285, 1935.

4. Toribara, T. Y., and Koval, L.: Determination of Calcium in Solutions Containing Ethylenediamine Tetraacetic Acid. (To be published.)
5. Terepka, A. R., Toribara, T. Y., and Dewey, P. A.: The Ultrafiltrable Calcium of Human Serum. II. Variations in Disease States and Under Experimental Conditions, J. Clin. Invest. **37**: 87, 1958.
6. Spencer, H. S., Greenberg, J., Berger, E., Perrone, M., and Laszlo, D.: Studies on the Effect of Ethylenediaminetetraacetic Acid in Hypercalcemia, J. LAB. & CLIN. MED. **47**: 29, 1956.
7. Spencer, H., Vankinscott, V., Lewin, I., and Laszlo, D.: Removal of Calcium in Man by Ethylenediamine Tetra-Acetic Acid—A Metabolic Study, J. Clin. Invest. **31**: 1023, 1952.
8. Spencer, H.: The Use of Chelating Agents in the Study of Mineral Metabolism of Man, *in* Seven, M. J., and Johnson, L. A., editors: Metal Binding in Medicine, Philadelphia, 1960, J. B. Lippincott Co.
9. Foreman, H.: The Pharmacology of Some Useful Chelating Agents, *in* Seven, M. J., and Johnson, L. A., editors: Metal Binding in Medicine, Philadelphia, 1960, J. B. Lippincott Co.
10. Carr, M. H., and Frank, H. A.: Edathamil (EDTA)-Treated Patients, J. A. M. A. **160**: 1258, 1956.

Lipid patterns in Niemann-Pick disease

J. A. BALINT, M.B., M.R.C.P.,* W. L. NYHAN, M.D., PH.D.,**
PAUL LIETMAN, M.D.,*** and D. A. TURNER, PH.D.****
Baltimore, Md.

A case of Niemann-Pick disease is described. The purpose of this study was to analyze the lipids of tissues obtained in vivo, using silicic and gas-liquid phase chromatography. Previous findings of the accumulation of sphingomyelin and free cholesterol in the liver have been confirmed. A fall in red blood cell cephalin and an apparent rise in plasma cerebrosides were also noted. Palmitic acid was the predominant fatty acid of the liver sphingomyelin, while the red cell cephalin, as compared with the normal, showed a decreased content of arachidonic acid and an increase in stearic acid.

In 1914 Niemann[1] published his now classic description of "Irene D." Pick[2] subsequently added the detailed pathologic description, and established the clear differentiation between this condition, now known as Niemann-Pick disease, and Gaucher's disease. Bloom and Kern[3] demonstrated that the excess tissue lipid was a phospholipid, and Klenk[4] showed this to be sphingomyelin. Crocker and Farber[5] recently have reviewed the literature of this condition and described 18 cases of their own. No results of chemical analysis using the new techniques of silicic acid and gas-liquid chromatography have been reported thus far. We have been fortunate in having been able to study the lipid composition of certain tissues obtained by surgical biopsy from a patient with Niemann-Pick disease. This paper represents the results of our studies.

From the Biochemistry Research Division, Department of Medicine, Sinai Hospital, Baltimore, Md.
Supported by the United States Public Health Service (Training Grant HTS 5399), and by the Nutrition Foundation, Inc.
Received for publication Jan. 12, 1961.
*Assistant Professor of Medicine, University of Alabama Medical Center.
**Assistant Professor of Pediatrics, Johns Hopkins University School of Medicine.
***Assistant Resident in Pediatrics, Johns Hopkins Hospital.
****Director, Biochemistry Research Division, Sinai Hospital, Baltimore, Md.

Case report

History. Patient P. K. (JHH 867610), a girl, was admitted for the first time to the Harriet Lane Home at the age of 8 months because of hepatosplenomegaly.* Rhinorrhea and noisy respirations were noted shortly after birth and persisted in spite of antibiotic therapy. Splenomegaly was noted first at 1 month and hepatomegaly at 8 months of age. The size of both affected organs increased markedly over the next 3 months. The patient had begun to smile socially at 2 months of age and to sit alone at 6 months, but by the age of 7 months she lost some steadiness in the sitting position, and the loss appeared progressive thereafter. Vision and hearing were regarded as normal. The history was otherwise unremarkable. Her mother and father are Americans of relatively undiluted Norwegian and German lineage, respectively. Two older siblings are in normal health.

Physical examination. On admission the patient appeared well developed and well nourished but chronically ill. Physical examination revealed the following: Temperature 37.4° C.; pulse 120; respirations 32 per minute; height 75 cm.; weight 9.6 kilograms; and head circumference 45.5 cm. Her hair was quite blonde and fine; her skin was very white but not suggestive of anemia. A broad nasal bridge, copious nasopharyngeal secretions, and the attendant noisy respirations contributed to a rather odd facial appearance which did not suggest any specific syndrome. She sat poorly without support, preferring to lie supine, although she could move both arms and legs with vigor. However, motor strength and reflexes were diminished throughout. Intellectual development and sensory examination were normal. A horizontal nystagmus was greater in the right than in the left eye. Pupils were round, regular, equal, and reacted to light. Vision could not be tested adequately, but opticokinetic nystagmus with large test squares indicated at least 20/200 vision. No corneal lesions were seen with the slit lamp, but small white deposits were seen in the posterior lens capsules bilaterally. Cherry red spots were seen in both maculas.

The abdomen was slightly protuberant. The liver extended 6 cm. below the right costal margin and was firm and smooth, with a sharp edge. The spleen extended 6 cm. below the left costal margin and was also firm and smooth. Physical examination showed the patient to be otherwise normal.

Roentgenographic examination revealed patchy increased density in both lung fields and hepatosplenomegaly. There was osteoporosis with flaring of the distal ends of the femora. The spongiosa in the upper end of the humerus and distal ends of the femora appeared poorly delineated in places and coarsened in others. Examination of the skull and lumbar spine showed them to be normal, as were the results of an intravenous pyelogram. Electroencephalography revealed a normal sleep record.

Laboratory examination. Laboratory examination revealed the following: Hemoglobin 12.0 Gm. per 100 ml.; hematocrit 36.5; mean corpuscular hemoglobin concentration 33 per cent; reticulocytes 1.2 per cent; W.B.C. 14,950 per cubic millimeter, with 68 per cent polymorphonuclear neutrophils, 55 per cent lymphocytes, 5 per cent eosinophils, and 1 per cent basophils. Several vacuolated mononuclear cells were seen in the peripheral smear. Bone marrow aspirated from the tibia contained typical Niemann-Pick cells. Urinalysis was normal. Pneumococci were isolated from the nasopharynx and Klebsiella from the throat. Rectal cultures were negative. Analysis of the serum revealed the following, all of which are expressed in milliequivalents per liter, unless otherwise indicated: Na, 146; K, 5.1; Cl, 110; Co_2, 23; serum urea nitrogen, 29 mg. per 100 ml.; Ca, 10.2; P, 4.6; and uric acid, 5.6 mg. per 100 ml. The total serum proteins were 6.3 Gm. per 100 ml., and the albumin and globulin were 4.1 and 2.2 Gm. per 100 ml., respectively. Prothrombin time was 13 seconds (100 per cent). The cephalin flocculation was negative and the thymol turbidity, 6.3 units. The alkaline phosphatase

*Referred by Dr. Harold Fink of Washington, D. C.

was 14.2 units (Bodansky's method), and the acid phosphatase (glycerophosphate substrate), 0.7 unit. The SGOT was 91 and the SGPT 60 units. The total serum lipids were 1,310 mg. per 100 ml., the phospholipids, 300 mg. per 100 ml., and the cholesterol, 410 mg. per 100 ml.

Surgical biopsy of the liver (Fig. 1) revealed liver cells and Kupffer cells containing foamy material consistent with the diagnosis of Niemann-Pick disease. Sections of skeletal muscle, subcutaneous tissue, and skin were normal.

Course. During 7 months of observation the liver and spleen enlarged to a level 10 cm. below the costal margins, occupying much of the abdomen. Neurologic loss was progressive. Deep tendon reflexes disappeared, legs became flaccid, and the muscles of the legs atrophied. The patient became unable to sit, crawl, or roll over. The upper extremities were not strong, but she could hold a toy or a bottle. Vision and hearing appeared to remain intact during this interval.

At 10 months of age she developed a leukocytosis of 42,500 per cubic millimeter, with a relative lymphocytosis in the absence of infection or acute change in her condition. This leukocytic response persisted for 3 months, after which leukocyte counts were within normal limits. She had frequent upper respiratory infections, but none were severe. A course of prednisone (25 mg. 4 times daily for 2 months, then decreased over 3 months) was without apparent effect on her condition, nor was a subsequent course of therapy with 5-fluorouracil and a later course of uracil mustard. At 13 months of age the total serum lipids were 1,920 mg. per 100 ml., and the phospholipids and cholesterol were 417 and 460 mg. per 100 ml., respectively. The SGOT had risen to 170 units, and the SGPT to 100 units.

Materials and methods

Approximately 2 Gm. of liver was excised at laparotomy prior to steroid therapy. At the same time a portion of subcutaneous fat and rectus muscle were also obtained. The tissues were packed into dry ice immediately. Subsequently, the tissues were cut into small pieces while still frozen, freeze-dried for 24 hours, weighed, and ground in a tissue grinder in 2:1 chloroform-methanol. The lipids were extracted in 20 vol. of chloroform:methanol (2:1 v/v) overnight; the extract washed with saline and then dried over sodium sulfate.

Samples of blood were obtained in heparinized tubes, in the fasting state 2 days preoperatively, immediately after operation, and 20 days later. The cells were separated by centrifugation and the separated plasma and cell volumes measured. Extraction was carried out as described, except that the cells were mixed with Celite to prevent coagulation by the solvent.

Aliquots of the chloroform extract of each tissue were used for the gravimetric estimation of the total lipid content. The total neutral lipid and total phospholipid fractions were determined similarly after separation on silicic acid.

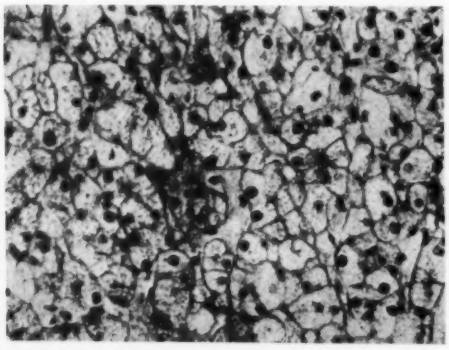


Fig. 1. Surgical biopsy of the liver. This shows the presence of liver cells and Kupffer cells filled with foamy material. (Original magnification ×400.)

Silicic acid* was prepared according to the method of Horning and associates.[6] This involves 4 initial washings with 3 N HCL followed by up to 20 washings with demineralized water until the pH rises to that of the water. This is followed by several washings in redistilled acetone. The silicic acid is dried for 16 hours under infrared lamps and is then sieved. The 100 to 200 mesh fraction is further dried in a vacuum oven at 50° C. for 2 hours at 20 mm. Hg. The dried silicic acid is kept in a ground-glass stoppered bottle.

The neutral lipids were separated from the phospholipids on 10 Gm. of silicic acid. The neutral lipid fraction (containing cholesterol ester, cholesterol, tri-, di-, and monoglycerides, and free fatty acids) was eluted with 300 ml. of chloroform, and the phospholipids were eluted in 300 ml. methanol. Each fraction was then taken to dryness in vacuo at 40° C.; the neutral lipid was dissolved in a known volume of hexane, the phospholipids were dissolved in chloroform.

The neutral lipids were chromatographed on 15 Gm. silicic acid columns (18 mm. i.d.; 10 cm., height) using increasing concentrations of benzene in hexane followed by 100 per cent chloroform for elution of the various lipid classes.† Phospholipids were chromatographed on 5 Gm. silicic acid (in column of 10 mm. i.d. and 8 cm. height) using increasing concentrations of methanol in chloroform as eluent (Figs. 2 and 3). Flow rates up to 1.5 to 2.0 ml. per minute were used with good results. Automatic fraction collectors were used throughout. Neutral lipid elutions were monitored by the weighing of every fifth fraction or by spotting small (10 to 20 gamma) aliquots from alternate tubes on alcohol-washed filter paper and staining these with a 10 per cent solution of phosphomolybdic acid in absolute alcohol. Phospholipid runs were monitored by phosphorus estimations on every tube, a 10 per cent aliquot being used for the determination. The aliquots were drawn from each tube as soon as practicable so that all aliquots were taken by the time a run was completed. Both neutral and phospholipid peaks were pooled according to the results of monitoring.

Chemical determinations were carried out by methods in general use in this laboratory. Thus, glycerol content was determined by a modification of the method of Van Handel and Zilversmit[7] (sodium bisulfite was used instead of sodium arsenite, and thiourea was added at the end to remove excess iodine). Ester linkages were estimated by the method of Snyder and Stephens.[8] Phosphorus content was measured by Bartlett's[9] procedure, and cholesterol by Tsugaeff's method[10] (Hanel and Dam modification).

Paper chromatography of the phospholipids was performed on silicic acid–impregnated Whatman No. 1 filter paper in a diisobutylketone:acetic acid:water (40:25:5) ascending system at room temperature. Appropriate pure standards were run on the same strip with each sample. Spots were identified by their Rf values relative to the appropriate standard and by their staining reactions with Ninhydrin followed by phosphomolybdic acid and stannous chloride.

After adequate identification of each peak, the fatty acids were transmethylated in a 5 per cent solution of concentrated sulfuric acid in methanol with dimethoxypropane (lipid dissolved in 5 ml. methanol, 0.25 ml. concentrated H_2SO_4 + 0.2 ml. dimethoxypropane). The fatty acid composition was determined by gas-liquid phase chromatography using 6 foot columns packed with 80 to 100 mesh Chromosorb W‡ coated with 17 per cent (w/w) diethylene glycol succinate polyester as liquid phase, and an argon ionization detector.§ Fatty acids were identified by comparison of their retention times with those of known standards run under identical conditions. The relative percentage of each fatty acid was calculated by triangulation.

*Mallinckrodt Chemical Works, St. Louis, Mo.

†Six per cent benzene/hexane 100 ml.; 18 per cent benzene/hexane 150 ml. for cholesterol ester; 60 per cent benzene/hexane 300 ml. for triglycerides; 100 per cent benzene 150 ml. for free cholesterol, and 300 ml. chloroform for di- and monoglycerides. The free fatty acids are eluted with the triglycerides and free cholesterol.

‡Johns-Manville Co., New York, N. Y.

§Column conditions: voltage 1,500; column temperature 168° C.; cell temperature 205° C.; flashheater temperature 278° C.; pressure 15 pounds per square inch; flow rate 80 milliliters per minute.

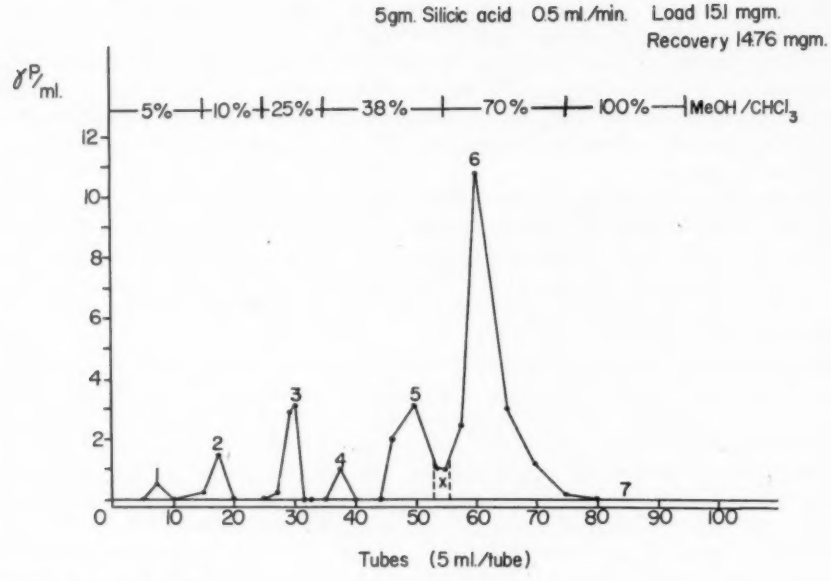


Fig. 2. Liver phospholipids.

Results

Table I presents the data on the dry weight of tissue obtained or the volume of plasma or cells, and their content of total neutral lipids and phospholipids. The high lipid content of both plasma and cells in the patient's blood, both in the "fasting" and the outpatient sample, requires comment. The latter was drawn 3 hours after a milk feeding, and both are in close agreement with results obtained on a spot specimen (see *Case report*). The

Table I. Lipid content of tissues

Tissue	*Dry weight*	*Total lipid (mg.)*	*Total neutral lipids (mg.)*	*Total phospho-lipids (mg.)*	*Total lipid (mg./100 mg. dry weight)**	*Neutral lipids (mg./100 mg. dry weight)**	*Phospho-lipid (mg./100 mg. dry weight)**
Liver	519.60 mg.	174.20	45.01	130.00	33.6	8.7	25.1
"Fasting" plasma	10 ml.	113.41	77.77	34.36	1,131.4	777.7	343.6
"Fasting" cells	5 ml.	39.85	17.54	22.41	797.0	350.8	448.2
Outpatient plasma	10 ml.	93.74	64.54	29.20	937.4	645.4	292.0
Postoperative plasma	10 ml.	29.66	20.54	9.43	296.6	205.4	94.3
Postoperative cells	10 ml.	10.75	6.24	4.14	107.5	62.4	41.4
Mother plasma	12 ml.	45.90	31.65	14.65	382.0	264.0	130.1
Father plasma	7 ml.	26.53	18.10	8.10	379.0	259.0	115.2
Adipose tissue	628.41	528.98	526.43	2.73	84.2	83.9	1.87

*Or per 100 ml. plasma or cells.

sample taken after operation is more likely to be a true fasting one, but it may have been affected by the stress of surgery. The most significant finding in these data, however, is the high lipid content of the liver (33.6 mg. per 100 mg. dry weight; normal is 15 to 18 mg. per 100 mg. dry weight) and the great preponderance of phospholipid over neutral lipid in this organ. (Normally, phospholipid accounts for 55 to 65 per cent of the total lipids.)

Analytic data on the major phospholipid fractions are given in Table II. Fig. 2 shows the elution pattern of the liver phospholipids. The great

Table II. Identification of major phospholipid fractions

Tissue	*Peak No.*	*Phosphorus ÷ ester ratio*	*Rf values*	*Standard Rf values*	*Ninhydrin*	*Phosphomolybdic acid*	*Conclusions*
Liver	2	—	0.651	0.660 S.C.	+	0	Cephalin
	3	—	0.654	0.660 S.C.	+	0	Cephalin
	5	—	0.579	0.580 S.L.	0	+	Lecithin
	6	—	0.473	0.477 S.S.	0	+	Sphingomyelin
Plasma	3	1:1.85	—	—	+*	0	Cephalin
	5	1:1.94	0.573	0.582 S.L.	0	+	Lecithin
	6	1:0.63	0.311	0.338 S.S.	0	+	Sphingomyelin
Red cell	3	1:1.65	0.742	0.738 S.C.	+	0	Cephalin
	5	1:1.87	0.665	0.627 S.L.	0	+	Lecithin
	6	1:0.44	0.496	0.496 S.S.	0	+	Sphingomyelin
Father plasma	3	1:2.04	—	—	+*	–	
	5	1:2.25	—	—	0	–	
	6	1:0.30	—	—	0	–	
Mother plasma	3	Insufficient for full tests			+*	0	Cephalin
	5	1:2.08	0.565	0.538 S.L.	0	+	Lecithin
	6	1:0.28	0.412	0.393 S.S.	0	+	Sphingomyelin
Normal red cell (S. R.)	3	1:1.98	—	— —	+	0	Cephalin
	5	1:2.15	0.84	0.81 S.L.	0	+	Lecithin
	6	1:0.64	0.60	0.45 S.S.	0	+	Sphingomyelin (contaminated with lecithin)
Normal plasma (S. R.)	3	1:2.19	—	— —	+*	0	Cephalin
	5	1:1.92	0.81	0.81 S.L.	0	+	Lecithin
	6	1:0.63	0.43	0.45 S.S.	0	+	Sphingomyelin
Normal red cell (D. S.)	3	1:1.66	0.801	0.72 S.C.	+	0	Cephalin
	5	1:2.36	0.667	0.638 S.L.	0	+	Lecithin
	6	1:0.4	0.520	0.529 S.S.	0	+	Sphingomyelin
Normal plasma (D. S.)	3	1:2.12	—	— —	+*	0	Cephalin
	5	1:1.97	0.646	0.638 S.L.	0	+	Lecithin
	6	1:0.34	0.544	0.529 S.S.	0	+	Sphingomyelin

S.C. = standard cephalin; S.L. = standard lecithin; S.S. = standard sphingomyelin.
*Ninhydrin test carried out in test tubes.

preponderance of peak 6 is clearly seen. It contains 57.5 per cent of the total phosphorus, and was identified as sphingomyelin. Peak 5 is lecithin, peaks 2 and 3 are cephalin, and peaks 1 and 4 remain unidentified. The fraction marked X was pooled separately to avoid contamination of peak 6 by peak 5.

The elution patterns of the outpatient plasma phospholipids are shown in Fig. 3 and Table IV. Separate runs of the fasting and the outpatient samples gave almost identical results. The predominant phosphorus peak in each run is the one in 38 per cent methanol in chloroform (peak 5). In the run illustrated, it accounts for 77.3 per cent of the total phospholipids, and was identified as lecithin. Peak 6 (70 per cent methanol) was identified as sphingomyelin. Peak 7, eluted in 100 per cent methanol, gave a phosphorus to ester (P:E) ratio of 1:0.75 and had an Rf value of 0.26 on paper chromatography. The spot gave a positive stain for choline; it is thought to be lysolecithin. Peaks 1, 2, and 3 contained insufficient material for proper identification, but peak 1 gave a positive anthrone reaction for carbohydrate. The "fasting" plasma phospholipid analysis revealed no phosphorus-containing material in the fractions eluted before the lecithin peak (38 per cent methanol in chloroform). However, these tubes, when pooled, were found to contain 3.4 mg. of material out of a total column load of 11.4 mg. (total recovery, 11.21 mg.). This material was anthrone-positive, Ninhydrin-negative, and contained no ester linkages. It is thought to be a cerebroside (cerebroside is barely detectable in normal plasma).

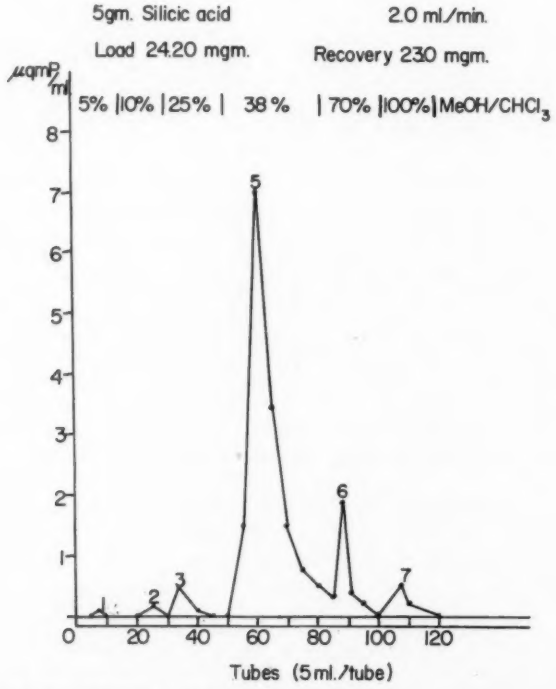


Fig. 3. Outpatient plasma phospholipids.

The results of the silicic acid chromatography of the patient's red cell phospholipids are shown in Table III, which also gives the data obtained from the red cells of a normal female child of 18 months of age (S. R.) and a normal male child of 7 months (D. S.). The gross reduction in the amount of cephalin in our patient's red cells (15 per cent of the total phospholipids) as compared to the normal control subject (27.2, 35.3 per cent, respectively), is evident.

Table III. Per cent composition of phospholipids of patient's red cells compared with those of 2 normal control subjects

Subjects	*Cephalin*	*Lecithin*	*Sphingo-myelin*	*Column load (mg.)*	*Recovery from column (mg.)*
Patient (P. K.)	15.0	43.5	28.1	22.7	20.4
Control (S. R.) 18 months old	37.2	39.8	19.5	8.38	8.23
Control (D. S.) 7 months old	35.3	32.8	27.9	6.28	4.94

Table IV. Per cent composition of plasma phospholipids of the patient, her mother and father, and 2 normal control subjects

Subjects	*Cephalin*	*Lecithin*	*Sphingo-myelin*	*Column load (mg.)*	*Recovery (mg.)*
Patient (P. K.)	3.7	77.2	12.9	24.2	23.0
Mother	5.6	54.3	27.6	15.7	14.65
Father	4.7	71.2	19.1	8.1	8.6
Control (S. R.) 18 months old	3.6	73.9	19.7	10.78	9.80
Control (D. S.) 7 months old	5.4	73.4	19.3	9.83	9.53

Table V. Per cent fatty acid composition of the liver sphingomyelin and red cell cephalin

Subject and substance	*Fatty acid*											*Total*
	C10	*C12*	*C14*	*C16*	*C16:1**	*C18*	*C18:1*	*C18:2*	*C18:3*	*C20*	*C20:4*	
P. K., liver sphingo-myelin	T†	T	0.7	53.2	—	28.6	1.1	7.0	—	T	8.9	99.5
P. K., red cell cephalin	T	T	2.7	33.8	T	20.0	18.2	9.2	T	—	10.6	97.2
S. R. (normal), red cell cephalin	T	T	3.7	37.6	T	10.5	20.4	5.6	T	—	21.6	99.4

*Number following colon is the number of double bonds.
†T = trace.

Table IV presents the results of the analysis of the plasma phospholipids of our patient's parents and 2 normal control subjects, as compared to those of the patient. No obvious differences are present.

Silicic acid chromatography of the neutral lipids of the outpatient plasma sample, the liver, and the adipose tissue did not reveal any noteworthy abnormalities except for an excess of free cholesterol in the liver (26.3 mg. out of total column load of 45 mg. 58.5 per cent). The relative proportions of the various fractions of the phospholipid of muscle appeared to be normal.

The results of the gas chromatographic analysis of the fatty acids of the liver sphingomyelin and red cell cephalin from the patient, as well as of the red cell cephalin of one normal child, are shown in Table V. The chief fatty acid in the liver sphingomyelin was palmitic acid (C_{16}) which represented 53.2 per cent of the total fatty acids. No lignoceric acid (C_{24}) was detected. Only a trace of behenic acid (C_{20}) was observed. Two major differences were noted in the fatty acid composition of the patient's red cell cephalin as compared to the normal: first, the relative increase in stearic acid (C_{18}) 20.0 per cent compared to 10.5 per cent in the normal, and second, the relative decrease in arachidonic acid (C_{20}:4), 10.6 per cent as compared with 21.6 per cent in the normal.

Discussion

In evaluating the results of these studies it is important to bear in mind some of the limitations of the methods used. Thus the cephalin fractions were not subdivided into phosphatidyl serine and ethanolamine, nor were the lecithin and sphingomyelin fractions analyzed quantitatively for choline or nitrogen content. Nevertheless, it is felt that the identification of the major fractions is sufficiently accurate for the present purpose.

Klenk[4] in his analysis of the phospholipids of the liver of a patient with Niemann-Pick disease, found that 24.7 per cent of the dry weight of the tissue was accounted for by crude sphingomyelin. In the present case, the total phospholipid was 25.1 per cent of the dry weight of liver and 57.5 per cent of this was sphingomyelin, thus the sphingomyelin was 14.4 per cent of the dry weight of the liver. Thannhauser and co-workers[11] found that phospholipids accounted for 37.1 per cent and sphingomyelin for 25.9 per cent of the dry weight of liver in a case of Niemann-Pick disease. In a normal liver the respective figures were 9.0 to 11.0 per cent and 0.3 to 0.5 per cent. These authors also found the serum phospholipid pattern to be normal. Therefore, the finding of a considerable amount of material, whose chemical properties strongly suggest that it is a cerebroside in the plasma phospholipids, is of interest in our case. These authors made no comment on the phospholipid pattern of the red cells. We have not been able to find any reference to a diminished cephalin content in the red blood cells in this condition. Reed and colleagues[12] found that cephalins accounted for 34 to 41 per cent of the lipid phosphorus of normal human red cells. This figure is in close agreement with the data obtained in our normal control subjects. The significance of the decreased cephalin in our patient's red cells is not clear.

Thannhauser and associates[11] found that, in their patient's liver, free cholesterol amounted to 4.5 per cent of the dry weight as compared to their normal figures of 0.4 to 0.5 per cent. Their patient was slightly older than ours, being 15 months of age at the time of death. The liver free cholesterol in our patient was 5.1 per cent of the dry weight, which is in close agreement with the findings quoted above.

Klenk[4] reported that the sphingomyelin isolated from normal liver as well as from tissues from Niemann-Pick disease contains palmitic (C_{16}), stearic (C_{18}), and lignoceric (C_{24}) acids. Thannhauser and associates [13, 14] considered that only 2 saturated fatty acids, palmitic and lignoceric, were present in normal sphingomyelin after removal of hydrolecithin. Klenk,[4] however, claimed that, in brain sphingomyelin from Niemann-Pick disease, stearic is almost the sole fatty acid. Thannhauser and Boncoddo[15] confirmed this finding, but also found traces of nervonic acid. They could find no lignoceric acid. Our data are not in agreement with their findings, as gas chromatography of the fatty acids of the liver sphingomyelin showed palmitic to be the predominant acid. The differences may, however, be due to tissue specificity. Furthermore, as we were able to identify our fatty acids only by their retention times on a single stationary phase (succinate polyester) these identifications cannot be taken as absolute, although the likelihood of error in the recognition of palmitic acid under these circumstances is small. There is no reference in the literature to the fatty acid composition of the cephalin fraction in Niemann-Pick disease. The relative increase in stearic acid associated with a corresponding decrease in arachidonic acid in our patient is therefore of interest.

We wish to thank Mrs. Ruth Vyner for technical assistance with the chromatographic and chemical analyses.

REFERENCES

1. Niemann, A.: Ein Unnbekanntes Krankheitsbild, Jahrb. f. Kinderh. **79**: 1, 1914.
2. Pick, L.: Niemann-Pick's Disease and Other Forms of So-Called Xanthomatosis, Am. J. M. Sc. **185**: 601, 1933.
3. Bloom, W., and Kern, R.: Spleens From Gaucher's Disease and Lipid Histiocytosis: Chemical Analysis, Arch. Int. Med. **39**: 456, 1927.
4. Klenk, E.: Ueber die Natur der Phosphatide in der Milz in Niemann-Pickscher Krankheit, Ztschr. f. physiol. Chem. **229**: 151, 1934.
5. Crocker, A. C., and Farber, S.: Niemann-Pick's Disease: A Review of 18 Cases, Medicine **37**: 1, 1958.
6. Horning, M. G., Williams, E. A., and Horning, E. C.: Separation of Tissue Cholesterol Esters and Triglycerides by Silicic Acid Chromatography, J. Lipid Res. To be published.
7. Van Handel, E., and Zilversmit, D. B.: Micromethod for the Direct Determination of Serum Triglycerides, J. Lab. & Clin. Med. **50**: 152, 1957.
8. Snyder, F., and Stephens, N.: A Simplified Spectrophotometric Determination of Ester Groups in Lipids, Biochem. et Biophys. Acta **34**: 244, 1959.
9. Bartlett, G. R.: Phosphorus Assay in Column Chromatography, J. Biol. Chem. **234**: 466, 1959.
10. Hanel, H. K., and Dam, H. Van.: Determination of Small Amounts of Total Cholesterol by the Tsugaeff Reaction; With a Note on the Determination of Lathosterol, Acta chem. scandinav. **9**: 677, 1955.

11. Thannhauser, S. J., Benotti, J., and Reinstein, H.: Studies on Animal Lipids; the Determination of Lecithin, Cephalin, and Sphingomyelin in Body Fluids and Tissues; With Analyses of Normal Human Sera. J. Biol. Chem. **129**: 709, 1939.
12. Reed, C. F., Swisher, S. N., Marinetti, G. V., and Eden, E. G.: Studies of the Lipids of the Erythrocyte. I. Quantitative Analysis of the Lipids of Normal Human Red Blood Cells, J. LAB. & CLIN. MED. **56**: 281, 1960.
13. Thannhauser, S. J., Benotti, J., and Boncoddo, N.: Isolation and Properties of Hydrolecithin (Dipalmityl Lecithin) From Lung; Its Occurrence in the Sphingomyelin Fraction of Animal Tissue, J. Biol. Chem. **166**: 669, 1946.
14. Thannhauser, S. J., Benotti, J., and Boncoddo, N.: The Preparation of Pure Sphingomyelin From Beef Lung and the Identification of Its Component Fatty Acids, J. Biol. Chem. **166**: 577, 1946.
15. Thannhauser, S. J., and Boncoddo, N.: The Chemical Nature of the Fatty Acids of Brain and Spleen Sphingomyelin. Isolation and Identification of Hydrolecithin From Brain and Spleen, J. Biol. Chem. **172**: 135, 1948.

A method for evaluation of disease and treatment in chronic leukemia

*Writing Committee**: S. WILLIAM ROSS, M.D., *Chairman*, ELEANOR J. MACDONALD, B.A., PHIL DAVIS, M.D., JAMES HAMMARSTEN, M.D., and WILLIAM C. LEVIN, M.D.

A new scoring method was devised to rate the severity of disease in patients with chronic lymphocytic and myelocytic leukemia. Weighted point values were assigned various manifestations of the disease. Values were arranged such that the greater the deviation from normal the higher the score. In each form of chronic leukemia the scores on the visit prior to death were analyzed on 20 consecutive patients. The final scores were significantly higher than the initial scores.

The scoring method was then used to evaluate 91 patients with chronic lymphocytic leukemia and 45 patients with chronic myelocytic leukemia entered into a therapeutic trials study. The initial scores of patients who died during the first 6 months of the study were significantly higher than those of the living patients. Therefore, on the basis of the initial score, all the patients were divided into 4 groups. During the first year of the study the cumulative survival for the population at risk was found to be inversely related to the mean initial score of each of the groups. It is concluded that this is a useful method for evaluating the status of patients with chronic leukemia.

In the treatment of disease, the goal of the physician is to relieve symptoms and disability, to prolong life and, whenever possible, to cure the patient. Unfortunately, there is no evidence that chronic leukemias can be cured by any

Supported by the Cancer Chemotherapy National Service Center of the National Cancer Institute, United States Public Health Service.

Received for publication Oct. 10, 1960.

*Committee of the Chronic Lymphocytic and Chronic Myelocytic Leukemia Protocal Sections, Southwest Cancer Chemotherapy Study Group, composed of principal investigators (above) and their associates at the following institutions: Baylor University College of Medicine (R. A. Hettig and Phil Davis); Lackland Air Force Base Hospital (C. B. Jackson, Jr.); Tulane University School of Medicine (C. C. Sprague and W. J. Stuckey); University of Arkansas Medical Center (S. William Ross and Otto M. Spurny); University of Texas M. D. Anderson Hospital and Tumor Institute (Daniel E. Bergsagel, C. C. Shullenberger, and Jess F. Gamble); University of Texas Medical Branch (W. C. Levin and Wayne Truax); University of Texas Southwestern Medical School (Eric E. Muirhead and W. W. Goldman); Veterans Administration Hospitals: Dallas, Texas (Roger H. Unger); Houston, Texas (J. B. Rochelle and R. M. Flasdick); Little Rock, Ark. (John C. Davis, Jr.); New Orleans, La. (Howard A. Buechner); Oklahoma City, Okla. (James Hammarsten and A. H. Woods).

Biostatistician: Eleanor J. MacDonald, University of Texas M. D. Anderson Hospital and Tumor Institute.

available agent. Treatment has been effective in the relief of symptoms and there is hope that life may be prolonged.[1] It is to these ends that therapy is presently directed. It would be helpful, therefore, to develop a standard method by which an accurate evaluation of the degree of disability and the stage of the disease could be made. Identification of the stages of the disease is of particular prognostic significance if, in the natural history of the disease, its course progresses from a mild to a severe form, terminating in death.

Since the median survival of patients with chronic lymphocytic leukemia is about 5 years and that of patients with chronic myelocytic leukemia is about 2.64 years, the measurement of therapeutic effectiveness as reflected by survival is a long and tedious process, albeit that it is the most important and meaningful index.[2] On the other hand, assessing therapeutic effectiveness by observing changes in individual manifestations of the disease is frequently invalid. In chronic lymphocytic leukemia, for example, several authors[3-5] have cited the lack of correlation between the level of the total leukocyte count and either prognosis or total amount of treatment required. Indeed, in one report of a case with a 29 year survival, the leukocyte count remained over 130,000 per cubic millimeter for 4 years and over 220,000 per cubic millimeter for 3 of these years without requiring treatment.[6] This case exemplifies the point that lowering the leukocyte count is not necessarily beneficial to the patient. Considering such limitations, methods used to evaluate the status of disease and response to therapy will be reviewed briefly.

Osgood and Seaman[7] and Reinhard and associates[2] have analyzed the effect of therapy in chronic leukemias primarily on the basis of survival. In addition, the influence of treatment on individual features of the disease was noted. Reinhard and associates[2] demonstrated differences between survival curves of patients with chronic lymphocytic and chronic granulocytic leukemia. Osgood and Seaman[7] compared survival curves in each form of chronic leukemia with survival curves of cases from collected series in the literature. The latter cases were treated by "conventional methods"; their own patients were treated with "titrated regularly spaced" total body irradiation. In each form of chronic leukemia the life expectancy was longer in the latter groups. The maximum difference in life expectancy was noted in the comparison of the 10 per cent of each group with the longest survival. This suggests that there are either different forms or different stages of the disease, but the authors did not attempt to classify their patients according to the stage of the disease except in retrospect and only according to survival. Hougie[8] reported 9 cases of chronic lymphocytic leukemia seen during early stages. He postulated that there may be no signs or symptoms in the first or "silent" stage, which may exist for many years. A second stage followed in which there were signs of the disease but no symptoms. This stage also may exist for many years. The third and most commonly recognized stage associated with classical signs and symptoms is considered to be the terminal phase and may last for as long as 20 years.

From an examination of 128 cases of chronic leukemia and a review of the literature of the subject, Pascucci[9] has listed those features which point to a

poor prognosis: (1) a very high or very low leukocyte count, (2) severe anemia, (3) a large number of "blast" forms, (4) excessively high or low platelet levels, (5) marked splenomegaly, (6) diffuse lymph node involvement, (7) presence of complications such as pneumonia, hydrothorax, tuberculosis, osteomyelitis, cardiovascular disease, etc., and (8) brief duration of symptoms prior to the diagnosis.

Rundles and co-workers[10] suggested 6 categories of response of chronic leukemia patients to therapy: excellent response, good response, fair response, questionable effect, no effect, and untoward effects of chemotherapy. Each category is defined in terms of functional status, symptoms, fever, hemoglobin, hematocrit, platelets, total leukocytes, per cent of lymphocytes or mature neutrophiles, and bone marrow findings. The limitation of this type of evaluation is apparent if it is considered that there are at least 3 forms of each category, in which case there would be 19,683 possible combinations, which would defy precise categorization. This method requires that each patient be rated by the consensus of experts and, hence, subjective bias is introduced. It is highly probable that these opinions will vary from time to time.

It is not unusual, following the treatment or during the natural history of chronic leukemia (particularly lymphocytic), for some manifestations of the disease to reflect improvement while others worsen or remain unchanged.[4] This feature makes evaluation of the patient's status particularly difficult. Therefore, it is proposed that a scoring method would be more objective and more valid if it expressed the summation of all manifestations of the disease, including the functional status of the patient. Such a method might be more accurate in reflecting significant change in the disease process and might reveal different stages of disease which would be of prognostic importance. It is the purpose of this paper to present and evaluate this type of scoring method.

Materials and methods

Each feature of the disease was examined and 2 questions were considered for weighting purposes. The first consideration was the actual significance of change in each feature with respect to functional status of the patient. For example, the level of the leukocyte count was considered to be of relatively minor importance, whereas the hemoglobin level was considered to be quite important. The activity status of the patient was assigned the greatest weight. The second consideration was the significance of change in each manifestation with respect to ultimate prognosis. This was a more difficult problem. The per cent of myeloblasts in chronic myelocytic leukemia, for example, is not invariably pertinent to the first consideration but it is to the second; it was therefore assigned more weight than the level of the total leukocyte count. Maximum numerical values were then assigned to each manifestation on the basis of its relative importance. The highest score was intended to represent the most prejudicial status. Changes within each manifestation were graded accordingly and numerical values were arbitrarily assigned. The score of zero implies a normal status. The highest value represents the most severe state of that manifestation. For example, a hemoglobin level of 12 Gm. per cent was rated as zero, while a hemoglobin value of less than 6.9 Gm. per cent was rated as 6. The scores assigned reflect the opinion of the group and are not proposed as absolute indices. Modification of the assigned scores was accomplished following statistical assessment. It is anticipated that additional modifications

will be required. The score assigned to a single manifestation applies only within the specific disease being studied and may be slightly or markedly different in other disease states. The weighted values allotted selected features of each disease are listed below.

Weighted point values allotted selected characteristics in chronic lymphocytic leukemia.

FEVER	VALUES
Days of fever in past 2 weeks	
Fever of outpatients would usually have one point	
No episodes	0
101° F. + fever less than 5 days	1
101° F. + fever 5 to 10 days	3
101° F. + fever 10 + days in 2 weeks	6
TRANSFUSIONS	
Each 500 ml. of blood	2
HEMOGLOBIN*	
12.0 + grams per cent	0
10.0 to 11.9 grams per cent	1
7.0 to 9.9 grams per cent	3
6.9 grams per cent or less	6
LYMPHADENOPATHY	
No lymphadenopathy is defined as statement by the examiner that no lymphadenopathy is present.	
No lymphadenopathy	0
Lymphadenopathy of 4.9 cm. or less	1
5.0 to 9.9 cm.	2
10.0 + cm.	3
PLATELETS*	
125,000 +	0
90,000 to 124,000	1
60,000 to 89,000	3
Less than 60,000	6
Over 3,000,000	3

SPLENOMEGALY	VALUES
No splenomegaly	0
Splenectomy	1
Enlarged to 9.9 cm.	1
Enlarged to 10.0 to 19.9 cm.	2
Enlarged 20.0 + cm.	3
HEPATOMEGALY	
No hepatomegaly	0
Less than 7 cm.	1
7.0 to 13.9 cm.	3
14.0 + cm.	6
TOTAL LYMPHOCYTES (ABSOLUTE)	
Less than 5,000	0
5,000 to 99,000	1
100,000 to 199,000	2
200,000 +	3
TOTAL LEUKOCYTES	
Less than 3,000	3
ACTIVITY STATUS	
Unrestricted	0
Home ambulant	3
Hospital ambulant	6
Bedridden, home or hospital	12
INFILTRATION	
Leukemic infiltration of skin without exfoliative dermatitis	3
Leukemic infiltration of skin with exfoliative dermatitis or leukemic infiltration of other nonreticuloendothelial structures	6

Weighted point values allotted selected characteristics in chronic myelocytic leukemia.

FEVER	VALUES
Days of fever in past 2 weeks.	
Fever of outpatients would usually have one point	
No episodes	0
101° F. + fever less than 5 days	1
101° F. + fever 5 to 10 days	3
101° F. + fever 10 + days in 2 weeks	6

SPLENOMEGALY	VALUES
No splenomegaly	0
Splenectomy	1
Enlarged to 9.9 cm.	1
Enlarged to 10.0 to 19.9 cm.	2
Enlarged 20.0 + cm.	3
HEPATOMEGALY	
No hepatomegaly	0

*If splenectomy is performed after entry into the study, the following is the procedure: If the patient improves after splenectomy (within 6 weeks) the minimal score will be the same as that immediately preceding splenectomy. If there is no improvement for 6 weeks and then improvement begins, the patient shall be scored as though he had no splenectomy.

TRANSFUSIONS	VALUES
Each 500 ml. of blood	2
HEMOGLOBIN*	
12.0 + grams per cent	0
10.0 to 11.9 grams per cent	1
7.0 to 9.9 grams per cent	3
6.9 grams per cent or less	6
PLATELETS	
125,000 to 1,000,000	0
1,000,000 to 3,000,000	1
Over 3,000,000	3
90,000 to 124,000	1
60,000 to 89,000	3
Less than 60,000	6
ACTIVITY STATUS	
Unrestricted	0
Home ambulant	3
Hospital ambulant	6
Bedridden, home or hospital	12

HEPATOMEGALY	VALUES
Less than 7 cm.	1
7.0 to 13.9 cm.	3
14.0 + cm.	6
TOTAL LEUKOCYTES	
Less than 15,000	0
15,000 to 49,000	1
50,000 to 299,000	2
300,000 +	3
Less than 3,000	3
MYELOBLASTS	
2.0 per cent or less	0
2.1 to 10.0 per cent	3
10.0 + per cent	6
INFILTRATION	
Leukemic infiltration of skin without exfoliative dermatitis	3
Leukemic infiltration of skin with exfoliative dermatitis or leukemic infiltration of other nonreticulo-endothelial structures	6

Four cases were selected from the studies of the Southwest Cancer Chemotherapy Study Group and will be described briefly. These cases illustrate the manner in which the scoring method reflects differences in response to treatment and how the total score correlates with some of the individual characteristics of the disease.

Case reports

Case No. 1. Patient A. H., a 63-year-old white man, was admitted to the study on November 7, 1958. The diagnosis of chronic myelocytic leukemia had been made in May, 1957. The patient had received Myleran from June to December, 1957, with an excellent therapeutic response. He was asymptomatic and received no further therapy until November 7, 1958, at which time the hemoglobin was 13.7 Gm. per cent, the total leukocyte count was 53,000 per cubic millimeter, with moderate immaturity of granulocytes, and the platelet count was 143,000 per cubic millimeter. Neither the spleen nor liver were palpable, and there were no other significant findings on physical examination. On that date, 6-mercaptopurine in a dose of 225 mg. per day was started. Fourteen days later, the total leukocyte count was 7,500 per cubic millimeter, the hemoglobin was 11.3 Gm. per cent, and the platelet count was 130,000 per cubic millimeter. During the next year, the dose of 6-mercaptopurine was adjusted at 2 week intervals in an attempt to maintain the leukocyte level below 15,000 per cubic millimeter. The dose necessary varied from 25 to 100 mg. per day. Even though the total leukocyte count was controlled fairly well, the immaturity of granulocytes increased during the last 6 months of life. During the last month of life, there was a rapid decrease in the hemoglobin level, a decrease in platelets, a rapid increase in the total leukocyte count, and a rise in the percentage of blasts in the peripheral blood. During the last 2 weeks of life, the spleen again became palpable.

*If splenectomy is performed after entry into the study, the following is the procedure: If the patient improves after splenectomy (within 6 weeks) the minimal score will be the same as that immediately preceding splenectomy. If there is no improvement for 6 weeks and then improvement begins, the patient shall be scored as though he had no splenectomy.

Table I A. Chronic lymphocytic leukemia: Comparison at 28 day intervals between 2 grou the other with initial scores of 11 and above

Days	*Hemoglobin*[1, 3]		*Leukocyte counts*[1, 4]		*Platelet counts*[1,5]	
	Observed contrasts A_1*/A_2†	*Value of p (if significant) (%)*	*Observed contrasts* A_1/A_2	*Value of p (if significant) (%)*	*Observed contrasts* A_1/A_2	*Value of p (if significant) (%)*
0	4/22	1	3/3	-	5/21	1
28	4/20	1	21/18	-	12/22	5
56	5/23	1	30/21	-	18/24	-
84	5/19	1	33/20	-	18/24	-
112	8/17	5	33/22	-	16/22	-
140	6/18	1	37/22	5	17/24	5‡
168	6/18	1	34/24	-	15/23	5
196	8/20	1	35/23	-	20/22	-
224	5/21	1	39/21	1	19/20	-
252	5/22	1	36/22	-	22/23	-
280	6/23	1	37/22	5‡	23/25	-
308	5/21	1	35/19	5	21/23	-
336	5/22	1	38/21	1	17/23	-

[1]Considering 49 patients with initial scores of 10 and below (N_1) and 42 patients with initial scores of
[2]Considering 48 patients with initial scores of 10 and below (N_1) and 40 patients with initial scores of
[3]Test of difference of hemoglobin at 9.9 Gm. per cent and below.
[4]Test of difference of leukocyte counts from 3,000 to 20,000 per cubic millimeter.
[5]Test of difference of platelet counts below 100,000 per cubic millimeter.
[6]Test of difference of hepatomegaly of greater than 7.9 cm. below the right costal margin in the mid-
[7]Test of difference in absence of lymphadenopathy between the 2 groups.
[8]Test of difference of splenomegaly of greater than 10.9 cm. below costal margin in the left midclavicular
*A_1 = number of patients with initial scores of 10 and below in group N_1.
†A_2 = number of patients with initial scores above 11 in group N_2.
‡Borderline.

The patient died as the result of a cerebrovascular accident on the three hundred and thirty-third day of the study, after having received a total dosage of 28,200 mg. of 6-mercaptopurine. At no time did the patient manifest evidence of toxicity of the drug. Postmortem examination revealed leukemic infiltration of the marrow, spleen, liver, and lymph nodes. There was encephalomalacia of the left cerebral hemisphere, pulmonary edema, and pulmonary embolism.

Clinically, the patient did well during the first 245 days of the study, but thereafter he deteriorated rapidly. This correlates well with the rise in the score from 1 on the two hundred forty-fifth day of the study, to 24 on the three hundred thirty-third day of the study (Fig. 1).

Case No. 2. Patient N. C., a 40-year-old Negro man, was admitted to the study on May 29, 1959. A diagnosis of chronic myelocytic leukemia had been made in June, 1958, at which time he was relatively asymptomatic with respect to the diagnosis of leukemia. In 1959, he reported epigastric distress and abdominal swelling for several months. Profound weakness which prevented him from working had existed for several weeks. Physical examination in May, 1959, revealed the patient to be chronically ill. The spleen was palpable 20 cm. below the left costal margin and 1 cm. to the right of the midline. The spleen was hard and not tender. The liver was moderately enlarged. There were no other significant physical findings. Laboratory examination revealed a leukocyte count of 700,000 per cubic millimeter with moderate immaturity of the granulocytes. The hemoglobin was 7.2 Gm. per cent and the platelet count was 246,000 per cubic millimeter. The marrow examination revealed an M:E ratio of 99 to 1. The patient was started on Myleran, 6 mg. per day, on May 29, 1959. Shortly thereafter a nodule on the skin of the right leg was noted, and biopsy

groups of patients in the study at least 12 months: One with initial scores of 10 and below,

Hepatomegaly[1, 6]		*Lymphadenopathy*[1, 7]		*Splenomegaly*[2, 8]	
Observed contrasts A_1/A_2	*Value of p (if significant) (%)*	*Observed contrasts* A_1/A_2	*Value of p (if significant) (%)*	*Observed contrasts* A_1/A_2	*Value of p (if significant) (%)*
7/11	–	6/0	–	9/7	–
4/11	5	4/0	–	6/6	–
7/12	–	6/2	–	6/6	–
4/12	5	7/2	–	3/7	–
8/11	–	5/2	–	2/7	–
8/11	–	10/3	–	1/7	–
6/12	–	10/1	5	3/7	–
8/12	–	6/2	–	3/7	–
6/12	–	9/1	5‡	3/6	–
6/12	–	7/1	–	3/7	–
6/12	–	9/1	5‡	3/6	–
7/12	–	10/0	1	3/7	–
6/12	–	9/0	1‡	3/7	–

11 and above (N_2).
11 and above (N_2).

clavicular line. (These measurements were made at the end of deep inspiration.)

line. (These measurements were made at the end of deep inspiration.)

revealed leukemic infiltration. There was gradual improvement until the one hundred eleventh day of the study, when the leukocyte count was 32,400 per cubic millimeter. The hemoglobin level had increased to 12.3 Gm. per cent and the platelet count to 430,000 per cubic millimeter. The liver was no longer palpable, and the spleen was palpable 4 cm. below the left costal margin on deep inspiration. There was no further decrease in leukocytes. The administration of 8 mg. of Myleran per day was begun on the one hundred eleventh day of the study. By the one hundred forty-sixth day, the leukocyte count was 11,500 per cubic millimeter, with little immaturity, and the platelet count was 235,000 per cubic millimeter. At this time, the hemoglobin was 11.2 Gm. per cent and the spleen was palpable 3 cm. below the left costal margin. The dosage of Myleran was reduced to 2 mg. per day, and thereafter there was an increase in the leukocyte count. The hemoglobin fell rapidly to 4.8 Gm. per cent by December 3, 1959, at which time the total leukocyte count was 325,000 per cubic millimeter with 90 per cent myeloblasts.

Myleran was discontinued and 6-mercaptopurine was started. The patient developed pneumonia, splenic infarcts, and worsening of the thrombocytopenia. Bleeding from the gastrointestinal tract appeared and he died on December 8, 1959. Postmortem examination revealed bilateral bronchopneumonia and leukemic infiltration of the spleen, liver, and kidneys.

This patient had been moderately ill on admission to the study, permitting good correlation with the moderately high admission score of 15 (Fig. 2). There was gradual improvement in the clinical status up to about the one hundred forty-sixth day of the study, as reflected by a gradual decrease of the score to 2. Thereafter, there was rapid deterioration in the clinical status associated with a rapid rise in the score. The patient died on the one hundred eighty-eighth day of the study.

Table I B. Chronic myelocytic leukemia: Comparison at 28 day intervals between 2 the other with initial scores of 11 and above

Days	*Hemoglobin*[1, 3]		*Leukocyte counts*[1, 4]	
	Observed contrasts A_1*/A_2†	*Value of p if significant* (%)	*Observed contrasts* A_1/A_2	*Value of p if significant* (%)
0	5/11	5	0/0	-
28	6/11	5	20/5	5
56	6/8	-	22/9	-
84	4/5	-	20/9	-
112	3/4	-	24/11	-
140	3/8	5‡	23/11	-
168	3/9	5‡	24/11	-
196	4/9	5‡	24/10	-
224	6/7	-	22/11	-
252	4/9	5‡	21/12	-
280	4/9	5‡	25/12	-
308	6/9	-	22/11	-
336	8/8	-	22/10	-

[1]Considering 28 patients with initial scores of 10 and below (N_1) and 17 patients with initial scores of
[2]Considering 28 patients with initial scores of 10 and below (N_1) and 16 patients with initial scores of
[3]Test of difference of hemoglobin at 9.9 Gm. per cent and below.
[4]Test of difference of leukocyte counts from 3,000 to 20,000 per cubic millimeter.
[5]Test of difference of splenomegaly of greater than 10.9 cm. below costal margin in the left mid-
[6]Test of difference of hepatomegaly of greater than 7.9 cm. below the right costal margin in the mid-
[7]Test of difference of platelet counts below 100,000 per cubic millimeter.
*A_1 = number of patients with initial scores of 10 and below in group N_1.
†A_2 = number of patients with initial scores above 11 in group N_2.
‡Borderline.

Case No. 3. Patient J. L., a 72-year-old Negro man, was admitted to the study on May 20, 1958. The diagnosis of chronic lymphocytic leukemia had been made in June, 1956, and at that time 20 mg. of nitrogen mustard was administered. He had been followed in the outpatient clinic from June, 1956, through May, 1958, with no symptomatic evidence of disease except splenic enlargement, moderate lymph node enlargement, and an elevated leukocyte count. On admission to the study, the liver was palpable 2 cm. below the right costal margin, and the spleen was palpable 3 cm. below the left costal margin. There was generalized enlargement of the lymph nodes with a mean diameter of about 1 cm. The leukocyte count was 160,000 per cubic millimeter. The marrow examination revealed the L:E ratio to be 98 to 2. This patient was treated with 1.2 mc. of P^{32} intravenously. There was reduction in the size of the liver but little change in the spleen or lymph nodes. The leukocyte count also fell, but there was little change in hemoglobin or platelet levels. The patient received injections of P^{32} in May, August, and October, 1958, and in August, 1959. During that time, minor fluctuations in hemoglobin and platelet levels were noted. The leukocytes fell fairly rapidly 2 months after the beginning of therapy. Thereafter, the leukocytes fluctuated between 10,000 and 20,000 per cubic millimeter. At the last observation, the patient was doing well clinically and the leukocyte count was 9,000 per cubic millimeter with 45 per cent lymphocytes, hemoglobin 11 Gm. per cent, and platelet count 140,000 per cubic millimeter. The spleen was still slightly enlarged.

This patient has been in the study 499 days, and during this period his clinical status has been good, requiring no limitation of activity. This correlates well with the total score, which has fluctuated between 1 and 6 (Fig. 3).

groups of patients in the study at least 12 months: One with initial scores of 10 and below,

Splenomegaly[1, 5]		*Hepatomegaly*[1, 6]		*Platelet counts*[2, 7]	
Observed contrasts A_1/A_2	*Value of p if significant (%)*	*Observed contrasts* A_1/A_2	*Value of p if significant (%)*	*Observed contrasts* A_1/A_2	*Value of p if significant (%)*
2/10	1	3/6	-	1/2	-
1/8	1	2/4	-	3/1	-
0/4	-	4/4	-	2/2	-
0/4	-	2/2	-	4/3	-
0/5	5	3/4	-	4/4	-
1/4	-	4/3	-	8/7	-
1/6	5	3/3	-	7/6	-
2/5	-	4/3	-	6/7	-
1/4	-	3/3	-	10/5	-
1/5	-	2/3	-	9/6	-
1/5	-	3/3	-	7/5	-
1/5	-	2/3	-	7/5	-
2/5	-	1/3	-	9/6	-

11 and above (N_2).
11 and above (N_2).

clavicular line. (These measurements were made at the end of deep inspiration.)
clavicular line. (These measurements were made at the end of deep inspiration.)

Case No. 4. Patient E. L., a 59-year-old white woman, was first seen at the participating institution and was entered into the study on November 12, 1958. The patient gave a history of the onset of ankle edema 4 or 5 months previously, and shortness of breath, fever, and cough for 6 weeks prior to admission. The patient was seen by a physician in the early part of November, 1958, at which time a diagnosis of chronic lymphocytic leukemia was made. Physical examination on admission revealed a firm nontender spleen, extending almost to the pelvic brim on the left. The liver was palpated about 2 cm. below the right costal margin and was slightly tender and moderately firm. Very large occipital, epitrochlear, and inguinal lymph nodes were present. The patient also had bilateral pleural effusion.

The pleural effusion was considered to be due to leukemic infiltration. Thoracentesis of 700 ml. of bloody fluid from the left pleural cavity was performed. The patient was started on 12 mg. of chlorambucil daily. At the time therapy was begun, the total leukocyte count was 384,000 per cubic millimeter. Chlorambucil was continued in the same dosage for a 3 month period with little or no change in the size of the liver, spleen, or lymph nodes. There was recurring pleural effusion, fever, and weight loss. The leukocyte count decreased gradually to 47,000 per cubic millimeter 3 months after the beginning of therapy. There was no increase in the hemoglobin, and there was a gradual reduction in the platelet count. The platelet count eventually fell to 19,000 per cubic millimeter and, at that time, the patient demonstrated extensive petechiae, ecchymoses, and bloody stools. Death occurred on the one hundred fourteenth day of the study. Postmortem examination revealed leukemic infiltration of the spleen, lungs, and cervical, axillary, thoracic, mesenteric, retroperitoneal, and inguinal lymph nodes. Bronchopneumonia and bilateral hydrothorax were present, but a subarachnoid hemorrhage was the immediate cause of death.

The patient was seriously ill on admission to the study and never demonstrated significant improvement. This correlates well with the score, which was initially high and remained high with little fluctuation, throughout the period of observation (Fig. 4).

Results and discussion

Patients used for the evaluation of the scoring method included 91 patients with chronic lymphocytic leukemia and 45 patients with chronic myelocytic leukemia observed in chemotherapy studies by the Southwest Cancer Chemotherapy Study Group between January, 1958, and June, 1959, who could have been in the study for 12 months. In Tables III A and III B, the 95 patients with chronic lymphocytic and the 54 patients with chronic myelocytic leukemia entered through October, 1959, were used and were considered at 6 months.

The data obtained on each patient in the studies were carefully examined and compared with the score of that patient at each 2 or 4 week interval. The data included the clinical and laboratory findings and general evaluation by the attending physician. The members of the evaluating committee were satisfied that each patient's score corresponded with the over-all appraisal of the patient.

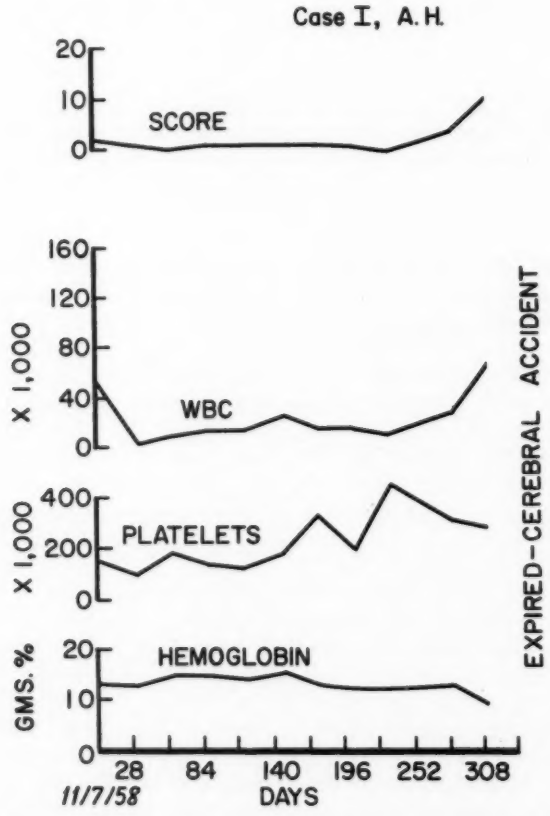


Fig. 1. Comparison of total score and leukocyte, platelet, and hemoglobin concentration in Case 1, a patient with chronic myelocytic leukemia with a low initial score.

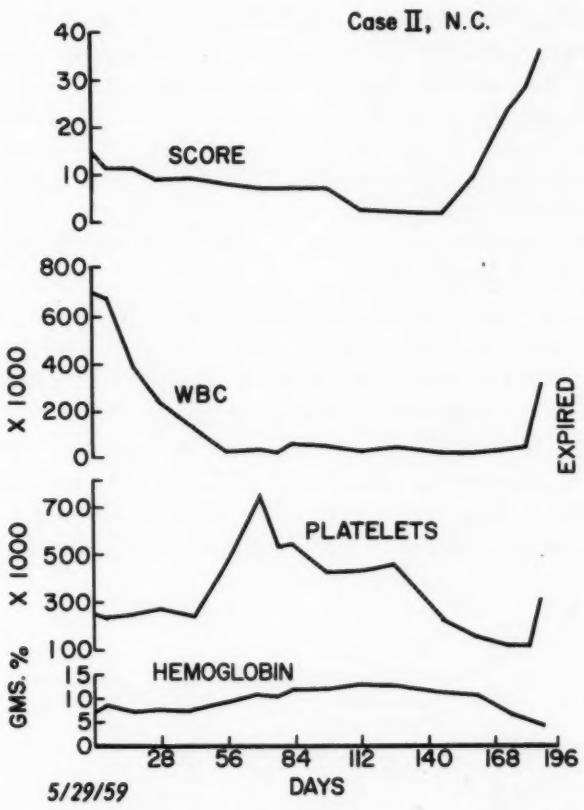


Fig. 2. Comparison of total score and leukocyte, platelet, and hemoglobin concentration in Case 2, a patient with chronic myelocytic leukemia with a high initial score.

At the various intervals, patients were divided into those with scores of 10 and below and 11 and above. The raw data of each manifestation did not differ consistently between the 2 groups. In chronic lymphocytic leukemia, the 2 groups did not differ significantly with regard to maintaining leukocyte counts between 3,000 and 20,000, nor to control of lymphadenopathy until the last few months of the observation period (Table I A). The patients with initial scores of less than 10 had a significantly higher hemoglobin level, more unrestricted activity, and were bedridden less than the other group (Tables I A and II). Thrombocytopenia was found more commonly in patients with initial scores of 11 or above, but did not differ significantly during the last 5 months of observation (Table I A). The degree of splenomegaly and hepatomegaly was not found to differ between the 2 groups (Table I A).

In chronic myelocytic leukemia, patients with initial scores of less than 10 were found to have higher hemoglobin levels and more unrestricted activity than the other group (Tables I B and II). There was a greater degree of splenomegaly in patients with scores of 11 and above on admission to the study but not during the last 5 months of observation (Table II). The total leukocyte

count, platelet count, and degree of hepatomegaly were not found to differ significantly between the 2 groups (Table I B). The hemoglobin level and activity status correlated best with the scores. The raw data of the other characteristics correlated inconsistently or not at all with the scores. This finding is compatible with the observation that, in a given patient, one manifestation of the disease may improve, another remain unchanged, and a third worsen simultaneously. The scoring method has the advantage of giving a relative measure of the sum of effects of all the features of the disease. To validate the scoring method, a number of hypotheses were tested.

For the method to be of value, a significant difference should be demonstrable between the average score of patients when first seen and their score in the terminal phase. To test this hypothesis, the statistician examined the records of 2 groups of 20 consecutive patients, one group with chronic lymphocytic leukemia and the other with chronic myelocytic leukemia, for each of whom adequate data for the scoring method was available. All had died at the M. D. Anderson Hospital before the inauguration of the study and had been treated by various "standard" methods. The deaths occurred between 1950 and 1955.

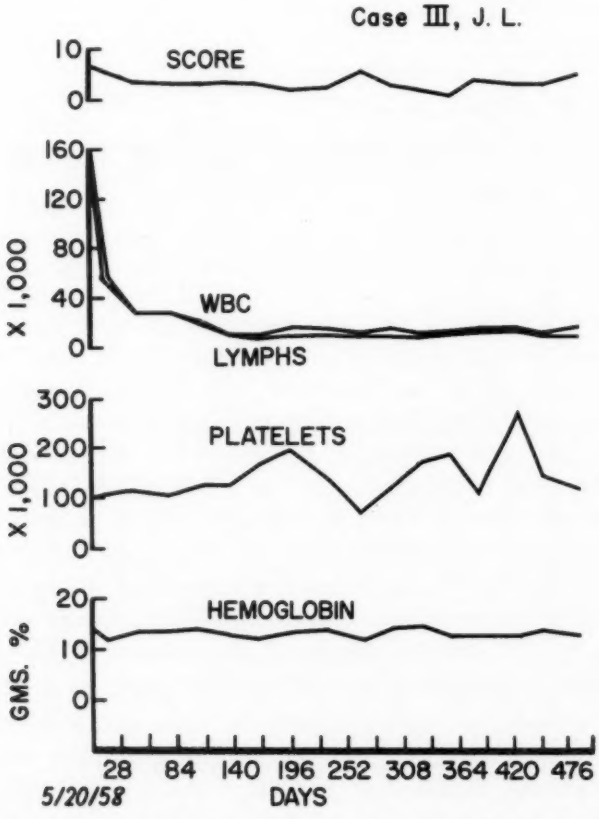


Fig. 3. Comparison of total score and leukocyte, lymphocyte, platelet, and hemoglobin concentration in Case 3, a patient with a low initial score with chronic lymphocytic leukemia.

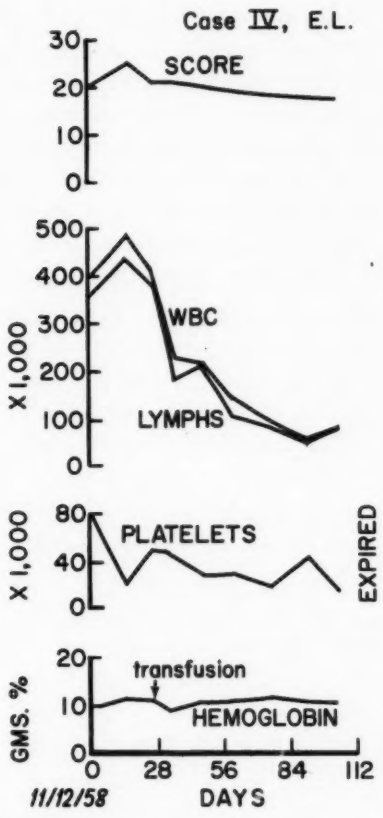


Fig. 4. Comparison of total score and leukocyte, lymphocyte, platelet, and hemoglobin concentration in Case 4, a patient with a high initial score with chronic lymphocytic leukemia.

The data in Tables III A and III B reveal that the mean initial score is significantly lower than the final mean score within each form. A similar comparison between the initial and the final scores of patients entered into each study is shown in these tables. In each form of chronic leukemia, the mean initial score of study patients who died is significantly lower than their mean final score. The mean final score at 6 months in patients who were still living was significantly lower than their mean initial score.

In chronic lymphocytic leukemia, the mean final score of the prestudy M. D. Anderson patients is significantly higher than that of the deceased study patients. This difference occurred because lower scores were assigned to patients confined to the home. In the M. D. Anderson prestudy group, all the patients died in the hospital, whereas many of the study patients died at home. The mean final score of the living study patients was considerably lower than those of both groups of dead patients. In chronic myelocytic leukemia, the mean initial score of the M. D. Anderson prestudy patients was significantly lower than that of those patients in the study who had died. This difference was not unexpected, since the dead M. D. Anderson patients included patients who were first seen

early in the course of their disease, whereas the dead study patients were all in the terminal phase, having been arbitrarily selected to include only patients who died within 6 months after entry into the study. This interpretation is supported by the observation that in chronic lymphocytic leukemia the mean initial score of the living patients is significantly lower than that of both groups of dead patients; and in chronic myelocytic leukemia, the mean initial score of the living patients is significantly lower than that of the dead study patients. These observations tend to validate the scoring method, since at least 2 forms or stages are differentiated in each type of leukemia.

It is reasonable to expect the amount of time patients have a given score to be inversely related to the level of the score if the scoring method grades the disease by severity. The data in Figs. 5*A* and 5*B* confirm this prediction. The

Table II. Activity status: Test of difference of activity status in 4 categories* between 2 groups with initial scores of 10 and below and 11 and above at 28 day intervals, of patients entered in the study 12 months and over

	Value of p (if significant)			
Days	*1* (%)	*2* (%)	*3* (%)	*4* (%)
A. Chronic lymphocytic leukemia				
0	1	-	1	1
28	1	-	1	1†
56	1	-	1	1
84	1	-	5	1
112	1	-	-	1
140	1	-	-	1
168	1	-	-	1
196	1	-	-	1
224	1	-	5	1
252	1	-	-	1
280	1	-	-	1
308	1	-	-	1
336	1	-	-	1
B. Chronic myelocytic leukemia				
0	1	-	-	-
28	1	-	-	-
56	-	-	-	-
84	-	-	-	-
112	5	-	-	-
140	-	-	-	-
168	-	-	-	-
196	5	-	-	-
224	1	-	-	5
252	5	-	-	5
280	-	-	-	-
308	5	-	-	-
336	5	-	-	-

*1, Unrestricted; 2, Ambulant, home; 3, Ambulant, hospital; 4, Bedridden.
†Borderline.

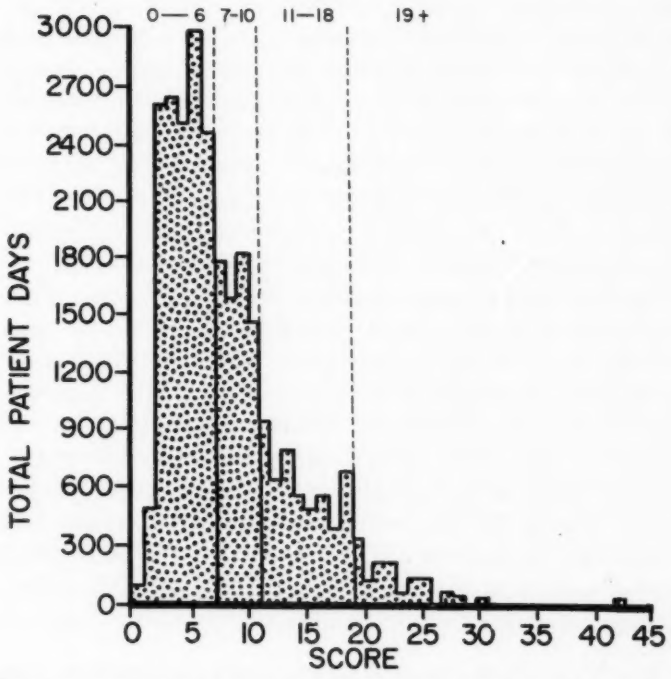


Fig. 5 A. Distribution of total patient days by scoring during 15 months of study of chronic lymphocytic leukemia; a total of 27,129 patient days for 104 patients.

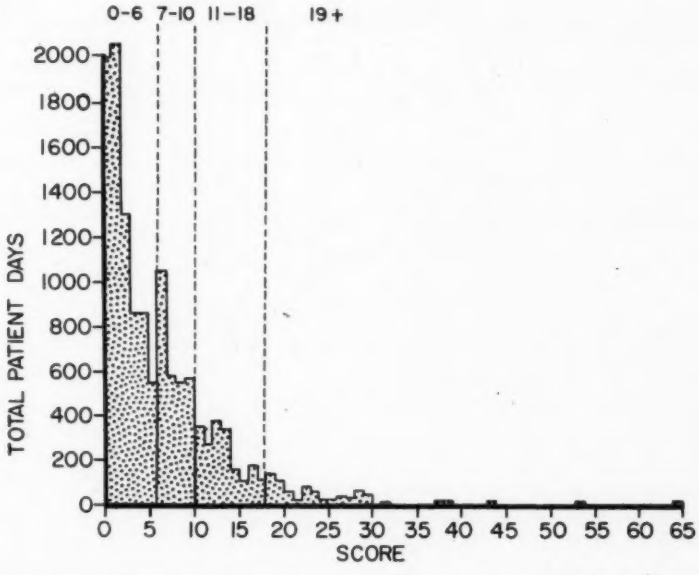


Fig. 5 B. Distribution of total patient days by scoring during 15 months of study of chronic myelocytic leukemia; a total of 13,340 patient days for 56 patients.

total number of patient days during 15 months of the study for each score is greatest for the low scores and becomes progressively shorter as the highest scores are approached. The scores are arbitrarily divided into 4 groups: viz. 0 to 6, 7 to 10, 11 to 18, and 19 and above, henceforth referred to as Group I, II, III, and IV, respectively, for each variety of leukemia.

Since the activity status of the patient was weighted considerably more than any other characteristic of the disease, it appeared as though the total scores would primarily reflect the patients' activity and that the effect of the other characteristics might be masked. To test this hypothesis, the total scores, including the activity status, were compared to the total scores minus the activity status in each group of patients at 3 month intervals. At each interval the quotients of the interval score divided by the initial score with the activity status included were compared with the quotients of the interval score divided by the initial score with the activity status excluded. At no time interval in any of the groups were these quotients significantly different. The p value was greater than 0.30 in all instances except in Group II at 6 months and groups II and III at 9 months in chronic lymphocytic leukemia and in Group III at 3 months in chronic myelocytic leukemia. Group III at 6 months in chronic lymphocytic leukemia had a p value between 0.1 and 0.2, whereas the others were all greater than 0.2.

Table III A. Chronic lymphocytic leukemia: Evaluation of initial and final scores of study compared with prestudy patients

Groups	*No. of patients*	*Mean initial score*	*Mean final score*
A. Prestudy deaths at M. D. Anderson Hospital	20	12.8 ± 7.1*	29.2 ± 5.3
Study patients			
B, Living	59	9.0 ± 4.9	6.6 ± 4.5
C, Dead	22	16.3 ± 7.4	21.7 ± 7.5
D, Treatment failures	14	11.8 ± 7.2	16.4 ± 8.2

t-Tests

	t	*p*
A (F) and B (F)†	17.10	.001
A (F) and C (F)	3.77	.001
C (F) and B (F)	5.21	.001
B (I) and C (I)‡	4.27	.001
A (I) and A (F)	8.28	.001
C (I) and C (F)	2.40	.02
B (I) and B (F)	2.76	.01
D (I) and D (F)	1.58	.1-.2
A (I) and B (I)	2.22	.05
A (I) and C (I)	1.56	.1-.2
B (I) and D (I)	1.38	.2-.3

*Standard deviation.
†(F) = Final.
‡(I) = Initial.

If the division of patients into 4 groups is to be clinically useful as a predictive tool then at least 2 other features should be noted. First, if patients are assigned to each group on the basis of their scores, the scores of each group of patients should continue to be different from each other in the same order when observed over a long period of time. Second, the death rate in each group during any given period of the study should be directly related to the initial mean or median score of each group. According to the initial score, patients were placed into one of the 4 groups.

Comparison of the mean scores of each group at 3 month intervals over a period of 12 months reveals a gradual increase at a similar rate (Figs. 6*A* and 6*B*). The only significant exception to this is seen in chronic myelocytic leukemia, with a marked drop of mean scores of Group IV during the first 5 months of therapy. The mean scores of these groups then climb to their previous high level and increase gradually thereafter.

Initially, the scores of patients with chronic lymphocytic leukemia in Groups I, II, III, and IV are significantly different from each other, as one might expect. At 12 months the scores of patients in Group IV are still statistically significantly different from the other groups and the scores in Group I are statistically significantly different from those of Group III. At the same time, Groups I and II differ with a nonsignificant p value of 0.1 and Groups II and

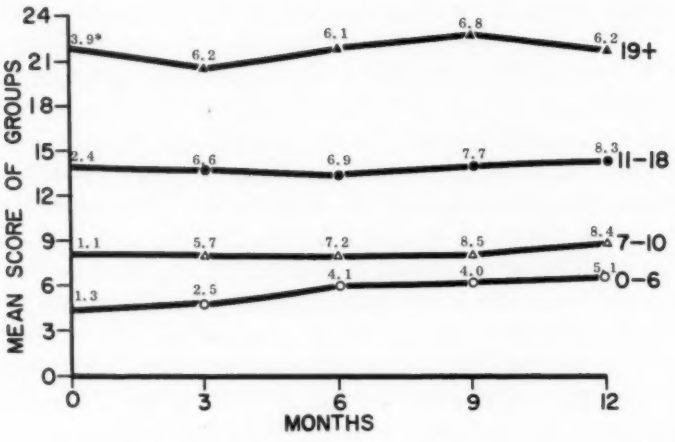
Table III B. Chronic myelocytic leukemia: Evaluation of initial and final scores of study compared with prestudy patients

Groups	*No. of patients*	*Mean initial score*	*Mean final score*
A, Prestudy deaths at M. D. Anderson Hospital	20	8.6 ± 5.7*	28.0 ± 6.6
Study patients			
B, Living	39	9.3 ± 4.7	5.6 ± 3.2
C, Dead	5	16.4 ± 2.3	24.2 ± 3.8
D, Treatment failure	10	10.2 ± 6.0	15.8 ± 9.1

t-Tests

	t	*p*
A (F) and B (F)†	14.35	.001
A (F) and C (F)	1.69	.1-.2
C (F) and B (F)	10.50	.001
B (I) and C (I)‡	5.55	.01
A (I) and A (F)	9.95	.001
C (I) and C (F)	3.94	.02
B (I) and B (F)	4.07	.001
D (I) and D (F)	1.62	.1-.2
A (I) and B (I)	.47	.6-.7
A (I) and C (I)	4.76	.01
B (I) and D (I)	.44	.6-.7

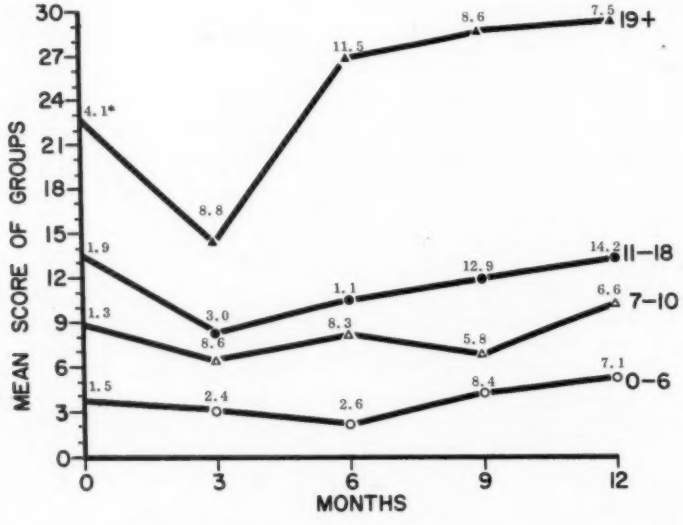
*Standard deviation.
†(F) = Final.
‡(I) = Initial.



*Standard deviation.

Fig. 6 A. Mean scores of patients with chronic lymphocytic leukemia divided into 4 groups according to initial scores and observed over a 1 year period.

III do not differ significantly. The scores of the 4 groups of patients with chronic myelocytic leukemia differ significantly from each other initially, but at 12 months only the score of Group IV differs significantly from the other groups. The standard deviation of the scores for each group at the end of 12 months has increased from twofold to sevenfold. This suggests that the scoring method does not sharply divide the chronic leukemias into stages or forms and that further modifications will be desirable.



*Standard deviation.

Fig. 6 B. Mean scores of patients with chronic myelocytic leukemia divided into 4 groups according to initial scores and observed over a 1 year period.

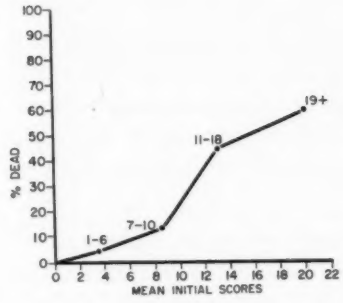


Fig. 7 A. Death rate during 15 months of a study of patients with chronic lymphocytic leukemia divided into 4 groups according to initial score.

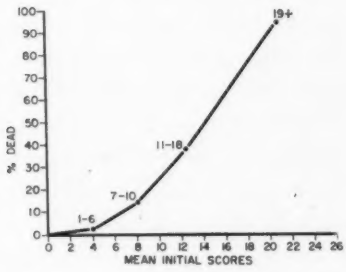


Fig. 7 B. Death rate during 15 months of a study of patients with chronic myelocytic leukemia divided into 4 groups according to initial score.

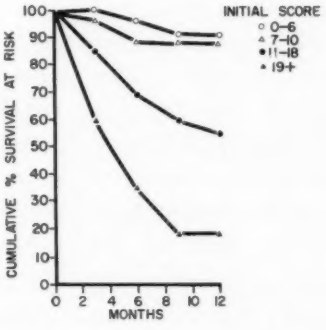


Fig. 8 A. Cumulative survival for population at risk in patients with chronic lymphocytic leukemia.

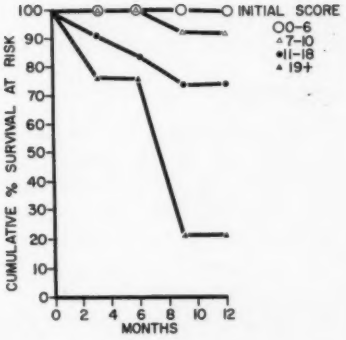


Fig. 8 B. Cumulative survival for population at risk in patients with chronic myelocytic leukemia.

Further validity to the predictive value of the scoring method is seen by comparing the mean score of each group with the per cent of patients in each group that died since the studies started (Figs. 7*A* and 7*B*). In chronic lymphocytic leukemia, approximately 5 per cent of patients in Group I died, whereas 60 per cent of Group IV had died. Similarly, in chronic myelocytic leukemia, about 2 per cent of patients in Group I died, whereas 100 per cent of patients in Group IV had died.

On the basis of population at risk the cumulative survival curves of all groups have different slopes[11] (Figs. 8*A* and 8*B*). In chronic lymphocytic leukemia, the cumulative survival at 12 months is 91.5 per cent for Group I and 18.0 per cent for Group IV. In chronic myelocytic leukemia, the cumulative survival at 12 months is 100 per cent for Group I and 25 per cent for Group IV.

In addition to predicting survival, the scoring method predicts how well the disease can be controlled after a given interval. In chronic myelocytic leukemia, at 12 months 62 per cent of Group I, 50 per cent of Group II, 22 per cent of Group III, and 0 per cent of Group IV had scores of 2 or less. At the same time, in chronic lymphocytic leukemia, 77 per cent of Group I, 47 per cent of Group II, 28 per cent of Group III, and 7 per cent of Group IV had scores of 7 or less.

Using the data reported by Rundles and associates,[10] Grizzle[12] found a general correlation between the 2 methods of evaluation. The average scores increased as the ratings from "excellent response" to "no effect" decreased.

This method of evaluation indicates that in both chronic lymphocytic and chronic myelocytic leukemia the 4 groups of patients, separated according to initial score, behaved differently under the conditions of the study. The patients in Groups I and IV usually maintained their respective low and high scores during a year of observation. Patients in Groups II and III had considerably more fluctuations in their scores during the same interval of time. This was noted even though the mean scores of these 2 groups did not change significantly. These data suggest that patients in Group I would not be good for therapeutic trial studies because of the benignity of the disease. Similarly, patients in Group IV appear to have irreversible changes and might yield results prejudicial against a drug that could be effective in patients who have less far advanced disease. This group, however, could be used for physiologic and toxicologic studies. Groups II and III would seem to be the more ideal for therapeutic trial studies because their course is subject to change.

REFERENCES

1. Karnofsky, J. N.: Panels in Therapy, Blood 10: 1060, 1955.
2. Reinhard, E. H., Neely, C. L., and Samples, D. M.: Radioactive Phosphorus in the Treatment of Chronic Leukemias: Long-Term Results Over a Period of 15 Years, Ann. Int. Med. 50: 942, 1959.
3. Lawrence, J. H., Law-Beer, B. V. A., and Carpender, J. W. J.: Chronic Lymphocytic Leukemia: A Study of 100 Patients Treated With Radioactive Phosphorus, J. A. M. A. 140: 585, 1949.
4. Osgood, E. E.: Panels in Therapy, Blood 10: 1062, 1955.

5. Shimkin, B. L., Lucia, E. L., Oppermann, K. C., and Mettier, S. R.: Lymphocytic Leukemia: An Analysis of Frequency, Distribution and Mortality at the University of California Hospital, 1913-1947, Ann. Int. Med. **39**: 1254, 1953.
6. Marlow, A. A., and Bartlett, G. R.: Survival for Twenty-Nine Years in Chronic Lymphocytic Leukemia, J. A. M. A. **152**: 1033, 1953.
7. Osgood, E. E., and Seaman, A. J.: Treatment of Chronic Leukemias: Result of Therapy By Titrated Regularly Spaced Total Body Radioactive Phosphorus or Roentgen Irradiation, J. A. M. A. **150**: 1372, 1952.
8. Hougie, C.: The Early Diagnosis and Natural History of Chronic Lymphatic Leukemia, Ann. Int. Med. **45**: 39, 1956.
9. Pascucci, L. M.: Chronic Leukemias: Statistical Study of Symptoms, Duration of Life and Prognosis, Radiology **39**: 75, 1942
10. Rundles, R. W., Grizzle, J., Bell, W. N., Corley, C. C., Frommeyer, W. B., Jr., Greenberg, B. G., Huguley, C. M., Jr., James, G. W., III, Jones, R., Jr., Larsen, W. E., Loeb, V., Leone, L. A., Palmer, J. G., Riser, W. H., Jr., and Wilson, S. J.: Comparison of Chlorambucil and Myleran in Chronic Lymphocytic and Granulocytic Leukemia, Am. J. Med. **27**: 424, 1959.
11. Berkson, J., and Gage, R. P.: Calculations of Survival Rates for Cancer. Proc. Staff Meet. Mayo Clinic, **25**: 270, 1950.
12. Grizzle, James: Personal communication.

Red cell transaminase levels in anemia. II. Thalassemia minor

MARTIN D. SASS, M.S., and PAUL W. SPEAR, M.D.
New York, N. Y.

The finding that red cell glutamic oxaloacetic transaminase activity is increased in populations of young cells has been applied to the study of patients with thalassemia minor and their families. Both the range of values and mean red cell glutamic oxaloacetic transaminase activity in 18 patients and affected members of 5 families were consistently above normal. No such increases were observed in a series of patients with and without anemia in a variety of diseases unassociated with markedly increased red cell proliferation.

In the first paper of this series[1] we presented data indicating that glutamic oxaloacetic transaminase (GOT) activity is increased in the red cells of patients with a variety of anemias characterized by bone marrow hyperplasia and increased red cell delivery. These findings suggested that the increased activity was associated with a decreased mean red cell age. Though reticulocytosis also could be demonstrated in association with the augmented enzyme activity, the latter usually persisted long after reticulocytosis diminished.

Later studies with tagged red cells carried out at this hospital,[2] as well as those of other investigators,[3] corroborated the view that increased enzyme activity could be demonstrated in young red cells as distinguished from reticulocytes. These findings made it reasonable to expect that increased red cell GOT activity might also reflect erythroid hyperplasia in association with minor decreases in erythrocyte survival. It is the purpose of this report to present preliminary results indicating that the measurement of this enzyme may have particular value in the study of thalassemia patients and their families.

Materials and methods

Patients selected for study were admitted to this hospital for treatment of a variety of complaints or were referred to us for evaluation. These included 5 families and 18 indi-

From the Medical Service of the Veterans Administration Hospital, Brooklyn, N. Y., and the State University of New York, College of Medicine, New York, N. Y.
Received for publication Oct. 13, 1960.

vidual patients with thalassemia, 6 patients with iron deficiency anemia of nutritional origin, and 9 patients with hemolytic anemia, both congenital and acquired. Controls included 13 hematologically normal subjects, all of whom exhibited hemoglobin levels ranging from 14.5 to 15.5 Gm. per cent. Additional control groups consisted of 30 patients with cardiovascular disease, with and without mild anemia, 15 with chronic infections, 10 with metabolic dyscrasias, and 24 with miscellaneous diseases without bleeding or hemolytic components.

Estimations of red cell GOT activity were performed on washed erythrocytes by a modification[4] of the spectrophotometric technique developed by Karmen.[5] For this determination, whole blood was collected in oxalate,[6] and the red cells were washed 3 times with saline and then reconstituted to the original volume. The red cell count and packed cell volume of this suspension were determined. A suitable aliquot was then diluted 1 to 20 with distilled water and allowed to stand for 10 minutes for complete hemolysis. After centrifugation at 2,500 r.p.m. for 10 minutes, the hemoglobin concentration of the supernatant was determined at 540 mμ in a Beckman DU spectrophotometer previously calibrated by the Van Slyke manometric determination of oxygen capacity. The GOT activity of a suitable aliquot of this hemolysate (0.1 to 0.3 ml.) was estimated by measurement of the rate of oxidation of diphosphopyridine nucleotide (DPNH) as outlined by Karmen.[5] A previously determined temperature correction curve served to correct all activities to a standard temperature of 25° C. This activity, in Karmen units, was then expressed as per either 100 mg. hemoglobin, one milliliter of packed cell volume, or 10^9 cells. Further centrifugation of the hemolysates at 40,000 r.p.m. (144,700 g. maximum) for 66 minutes did not result in any significant reduction in enzyme activity.

Results

Although our previous studies on red cell GOT activity were carried out on whole blood, it was realized that both plasma and white cells contributed a small but definite increment in enzyme activity ascribed to the red cells. Despite this, the mean activity in a group of patients with a variety of hemolytic anemias was some 2 to 2½ times greater than that of 2 control groups.[1]

Since it was expected that the results with thalassemia patients would be somewhat less clear-cut, both plasma and buffy coat were removed by saline washes before GOT activity was estimated. The discrepancy between the mean enzyme activity of our previous control groups[1] and those of our present study is probably due to this refinement in technique as well as to the increased sample size.

The distribution of individual enzyme values obtained in all 9 groups of patients is presented in Fig. 1. With the exception of the hemolytic anemia and the 2 thalassemia groups, 93 out of the 98 control values fell within the range of 600 to 1,600 units per milliliter of packed cells. Of the 4 patients whose red cell GOT values exceeded this range, 2 were of Mediterranean origin and 2 presented themselves with psoriasis and a fever of unknown etiology, respectively. The mean enzyme activity as well as the range of values of the thalassemia group are distinct from those of the various control subjects. Even more marked elevations are seen in the patients with hemolytic anemia and thalassemia major.

The results in those patients with iron deficiency anemia are of notable interest in view of the difficulty in distinguishing these from the congenital hypochromic, microcytic anemias. The present findings suggest that this parameter may be useful in this regard. Unfortunately, sufficient numbers of

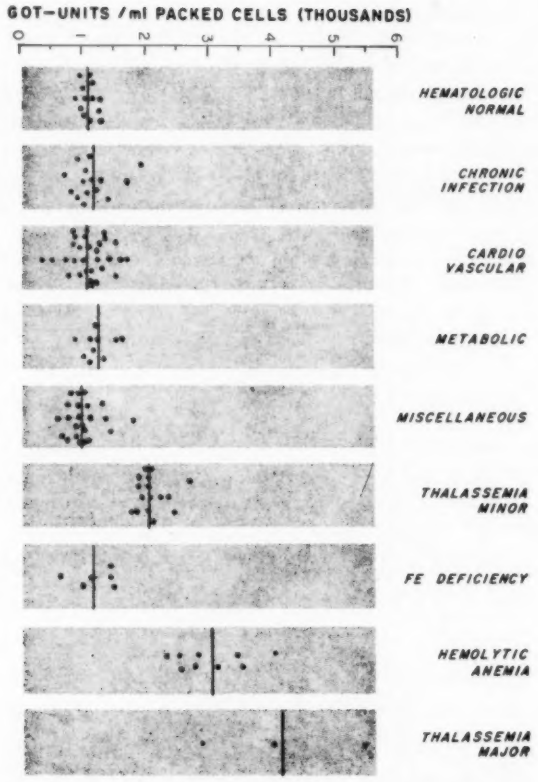


Fig. 1. Red cell transaminase activity in various diseases. Solid line represents the mean of each group.

patients were not available for study and this point must await further clarification.

In order to show that the microcytosis encountered in thalassemia is not in itself wholly responsible for the elevated values, the GOT activity of selected groups is presented in Table I on the basis of hemoglobin content, cell volume, and cell count. The contrast between the thalassemia and the control group is

Table I. Comparison of GOT activity

Group	*No.*	*Mean hemoglobin (Gm. %)*	*GOT activity*		
			Units per 100 mg. hemoglobin	*Units per ml. packed cells*	*Units per 10^9 cells*
Control	35	12.5	327 (120-480)*	1,022 (350-1,410)	94 (35-141)
Thalassemia minor	18	11.6	751 (460-960)	2,114 (1,480-2,720)	155 (126-313)
Iron deficiency	6	8.3	465 (380-520)	1,260 (740-1,590)	—

*Range indicated by parentheses.

quite clear irrespective of the basis for comparison. Furthermore, those patients with iron deficiency anemia exhibited normal red cell enzyme levels despite a microcytosis similar in degree to that seen in thalassemia minor. Some influence, however, can be detected. To avoid this, subsequent results are reported on the basis of cell count only.

To evaluate the correlation between this indication of increased red cell proliferation and other parameters of evaluation, additional studies were carried out on the families of several patients with thalassemia. In Family G (Table II) it is clearly apparent that the elevated GOT activity in the red cells of the father and Daughters Nos. 1 and 3 is consistent with the erythrocytosis, typical thalassemia morphology, hypochromic microcytosis, and increased fetal hemoglobin (normal: 3 per cent) found in these subjects.

In Family T, the increased red cell enzyme activity in the father and Daughters Nos. 1 and 2 is consistent with the majority of findings indicative of thalassemia despite the normal fetal hemoglobin levels. There are similar indications of correlation in the remaining families. The findings in Family Fe, however, deserve emphasis. The diagnosis of thalassemia major in the son was

Table II. Hematologic and enzyme studies on thalassemia families

Patient	*Million R.B.C. per mm.³*	*Hemoglobin (Gm. %)*	*MCV (cμ)*	*MCHC (%)*	*Typical morphology*	*Fetal hemoglobin (%)*	*GOT (units/10⁹ cells)*
Family G							
Father	5.9	13.8	76	31	Thalassemia	4.7	147
Mother	3.74	12.2	99	33	Normal	2.7	98
Daughter No. 1	4.77	10.6	—	—	Thalassemia	7.0	193
Daughter No. 2	4.75	11.7	75	33	Normal	2.7	81
Daughter No. 3	5.19	10.4	71	28	Thalassemia	12.0	146
Family T							
Father	5.65	11.0	83	23	Thalassemia	1.0	158
Mother	4.48	12.6	105	27	Normal	0.9	87
Daughter No. 1	5.78	12.2	66	32	Thalassemia	4.5	147
Daughter No. 2	4.85	9.4	80	24	Thalassemia	2.1	151
Family F							
Father	5.6	12.3	73	30	Thalassemia	0.8	136
Daughter No. 1	5.02	10.4	70	30	Thalassemia	5.8	147
Daughter No. 2	4.26	12.0	89	32	Normal	3.7	96
Family Tr							
Father	5.75	11.1	68	28	Thalassemia	2.3	158
Son	5.5	7.2	57	23	Thalassemia	7.1	126
Family Fe							
Father	5.5	12.9	76	31	Thalassemia	1.9	155
Mother	4.5	12.2	89	31	Thalassemia	2.1	149
Son*	3.7	5.4	60	25	Thalassemia	62.0	310

*Thalassemia major.

made on the basis of both clinical and hematologic findings. The mother, on the other hand, exhibited a normochromic, normocytic anemia without erythrocytosis or elevated fetal hemoglobin. Both the elevated GOT activity and the typical morphology were consistent with the expected appearance of the thalassemia gene in both parents.

The GOT activity found in this limited group of patients with thalassemia minor ranges from 126 to 193 units per 10^9 cells up to 300 units per 10^9 cells in the more severe thalassemia syndromes. In only 2 cases were the values for patients with thalassemia found to overlap the normal range. One wonders, however, whether it is correct to compare directly the enzyme activity of the small cells found in thalassemia with the larger normal erythrocytes of our control groups. If we correct the measured activity to a standard cell size of 90 $c\mu$ (the validity of which is at present under study) for both the control and thalassemia groups, the differences between the 2 groups become even more distinct.

Discussion

At present there is no clear understanding of the significance of either the increased levels of transaminase activity in younger cells or the progressive decrease in the activity of this as well as other enzymes that occurs as the erythrocyte matures. Granick[10] has postulated that this decrease may represent a "natural consequence of aging." Since this pattern is clearly not characteristic of all enzymes found in the erythrocyte,[3] it is possible that the increased activity of certain of these may be related to their specific role in synthetic or metabolic processes occurring in the immature cell. This consideration focused the emphasis on glutamic oxaloacetic transaminase, a pyridoxine linked enzyme, as the enzyme for study in view of the relationship between pyridoxine deficiency and hemoglobin synthesis. Similar emphasis might well be placed on other pyridoxine-linked enzyme systems involved in the synthesis of the heme rather than the globin moiety of the hemoglobin molecule.

Quite apart from speculations on the significance of increased enzyme activity in terms of the metabolism of the maturing erythrocyte, there appears to be sufficient evidence that increased enzyme activity in the peripheral blood is associated with the presence of increased numbers of young cells. This increase in thalassemia minor, as well as in the more frankly hemolytic syndromes, suggests a rate of red cell delivery somewhat greater than normal. It would be premature, however, to interpret this as a definite indication of decreased red cell survival, despite the reasonableness of such a conclusion. Studies on red cell survival with the Ashby[7] technique show that this conclusion is not supported by experimental evidence except in the more severe form of anemia.[8] However, the limitations of this technique in assessing minimal decreases in erythrocyte survival are not clear.

The practical application of these findings is in no way limited by the speculative nature of these implications. Certainly the series of thalassemia patients studied is much too small for definitive conclusions. However, there can

be no doubt that the differences in red cell GOT activity are clear and distinct. Certain questions remain to be answered. Included in these is the relationship of cell size or volume to enzyme activity. A direct relationship would permit the comparison of enzyme values on the basis of packed cell volume instead of the more inaccurate and laborious red cell count. We have found the former measure to be adequate for screening purposes. In those cases where values in excess of 1,600 units per milliliter of packed cells were obtained, subsequent determinations were carried out to permit expression of the enzyme activity on the basis of 10^9 cells.

It is possible that similar results might be obtained with one or more of a number of other enzymes already shown to be increased in reticulocytes and young cells. However, it is interesting that in the most thoroughly studied of these, cholinesterase,[9] only minimal increases were demonstrated in hemolytic anemias with reticulocytoses of 12.5, 20.0, and 31.0 per cent. In thalassemia minor no such increases could be demonstrated. Furthermore, determinations of transaminase activity recently have become almost routine. With slight modification, to allow for analysis of red cell hemolysates, this technique can provide further valuable information in distinguishing thalassemia in individual patients and their families.

REFERENCES

1. Sass, M., and Spear, P. W.: Whole Blood Transaminase Levels in Anemia, J. LAB. & CLIN. MED. **51**: 926, 1958.
2. Levy, L. M., Walter, H., and Sass, M.: Enzymes and Radioactivity in Erythrocytes of Different Ages, Nature **184**: 643, 1959.
3. Marks, P. A., Johnsen, A. B., and Hirschberg, E.: Effect of Age on the Enzyme Activity in Erythrocytes, Proc. Nat. Acad. Sc. **44**: 529, 1958.
4. Steinberg, D., and Ostrow, B. H.: Serum Transaminase as a Measure of Myocardial Necrosis, Proc. Soc. Exper. Biol. & Med. **89**: 31, 1955.
5. Karmen, A.: A Note on the Spectrophotometric Assay of Glutamic-Oxalacetic Transaminase in Human Blood Serum, J. Clin. Invest. **34**: 131, 1955.
6. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, J. LAB. & CLIN. MED. **19**: 777, 1934.
7. Hamilton, H. E., Sheets, R. F., and DeGowin, E. L.: Studies With Inagglutinable Erythrocyte Counts. II. Analysis of Mechanism of Cooley's Anemia, J. Clin. Invest. **29**: 693, 1950.
8. Kaplan, E., and Zuelzer, W. W.: Erythrocyte Survival Studies in Childhood. II. Studies in Mediterranean Anemia, J. LAB. & CLIN. MED. **36**: 517, 1950.
9. Sabine, J. C.: Erythrocyte Cholinesterase Titers in Hematologic Disease States, Am. J. Med. **27**: 81, 1959.
10. Granick, S.: The Chemistry and Functioning of the Mammalian Erythrocyte, Blood **4**: 404, 1949.

Red cell transaminase levels in anemia. III. Acute and chronic blood loss

MARTIN D. SASS, M.S., and PAUL W. SPEAR, M.D. *New York, N. Y.*

Red cell glutamic oxaloacetic transaminase activity has been shown to increase markedly in otherwise normal patients within 10 to 14 days after an episode of acute blood loss. This increase, persisting for at least 6 weeks, reflected hemorrhage more reliably than reticulocyte response. Even greater increases in glutamic oxaloacetic transaminase activity were demonstrated in 70 per cent of a group of patients with anemia associated with chronic blood loss. In these patients there was a rough inverse correlation between extent of blood loss and increase in red cell glutamic oxaloacetic transaminase activity.

Glutamic oxaloacetic transaminase (GOT) activity already has been shown to be elevated in the red cells of patients with a variety of anemias characterized by an increased rate of delivery of red cells into the peripheral blood.[1] In the companion paper of this series we have presented data indicating that in thalassemia minor, red cell GOT activity is similarly increased, suggestive of a decreased mean cell age.[2]

In these studies increases in enzyme activity were demonstrated mainly in congenital and acquired hemolytic anemia as well as in megaloblastic anemia during therapy. Despite contraindications, it is conceivable that these findings reflect an altered metabolism characteristic of the abnormal red cells found in these syndromes. Similarly, the erythrocytes of patients with chronic blood loss anemia, though presumably normal, could reflect abnormal metabolic patterns resulting from the continued loss of iron and other heme building materials.

It is the purpose of this report to add to the limited data previously reported in chronic blood loss anemia and to present the findings in a group of otherwise normal patients after an episode of acute hemorrhage. Apart from the stress of a mechanically induced anemia, which in itself might produce abnormality, this latter clinical state appears to be the closest approximation to the normal, together with an increased rate of proliferation of erythrocytes.

From the Medical Service of the Veterans Administration Hospital, Brooklyn, N. Y., and the State University of New York, College of Medicine, New York, N. Y.

Received for publication Oct. 13, 1960.

Materials and methods

Subjects studied included 79 patients with no hematologic abnormality (Group A), 27 with a history of chronic blood loss of varying degree (Group B), 7 with gastrointestinal disease and no anemia (Group C), and 12 patients admitted during or immediately following acute hemorrhage of the gastrointestinal tract (Group D). Of Group D, 10 patients exhibited moderate anemia with hemoglobin levels slowly rising over the entire period of study. Five of these had 3 to 4 + stool guaiacs for a limited period not exceeding 3 days after admission. Five had a history of melena immediately preceding hospitalization. Of the remaining 2 patients in this group, one was admitted with positive stool guaiacs and a normal hemoglobin level. The other gave a history of melena immediately preceding admission, together with slight anemia. All other laboratory findings were normal. No history of chronic bleeding could be elicited in any of these cases.

Blood was drawn in dry oxalate for the determination of hemoglobin and red cell transaminase. Reticulocyte counts also were done throughout the entire period of study. The determination of red cell GOT activity was carried out as described in the preceding paper.[2] Results are expressed in units per milliliter of packed cells.

Results

Fig. 1 presents the distribution of the levels of red cell GOT activity over a variable period after bleeding obtained in 9 out of the 10 patients with moderate anemia in Group D. The normal range represents 2 standard deviations above and below a mean of 1,130 units per milliliter of packed cells obtained in a group of 79 control patients (Group A). It is apparent that all values fell within these limits up to a period of from 10 to 14 days after the estimated onset of hemorrhage. Subsequently, red cell GOT levels were elevated variably with some indication of a return to normal after approximately 45 days. The complete

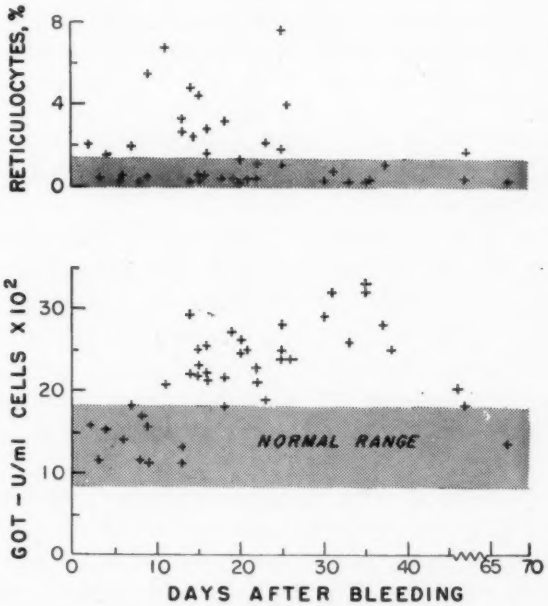


Fig. 1. Distribution of red cell transaminase activity and reticulocyte counts in 9 cases of anemia secondary to acute blood loss.

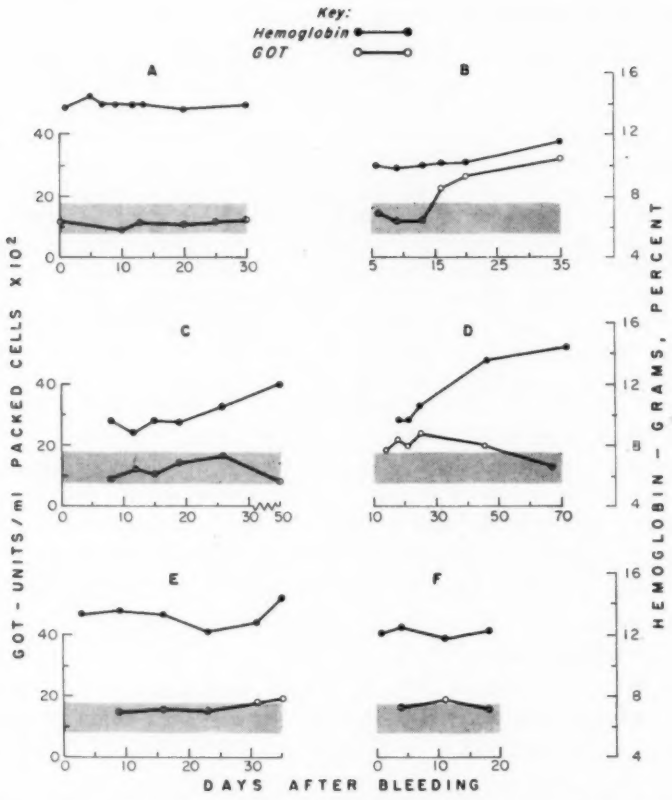


Fig. 2. Serial measurements of red cell transaminase activity and hemoglobin in selected representative patients (see text). Shaded area represents the normal range of red cell GOT activity.

absence of any values within the normal range between 14 to 45 days is in sharp contrast to the occurrence of both normal and elevated reticulocyte counts throughout the period of study.

Representative data on individual patients in whom serial estimations of red cell GOT activity and hemoglobin level were carried out are presented in Fig. 2. When a normal patient with no evidence of bleeding was followed over a period of 30 days (Fig. 2, *A*), relatively constant levels of both hemoglobin and GOT were observed. Analysis of the variations in enzyme activity encountered in a group of normal patients indicated that an increase of 260 units per milliliter of packed cells would represent a significant change (2.5 standard deviation).

Nine of the 10 patients with moderate anemia showed marked changes in red cell GOT levels by the fourteenth day after bleeding had occurred (Fig. 2, *B*). This high level had returned to within the normal range between the forty-fifth and sixty-fifth day (Fig. 2, *D*) in those patients who remained available for study. One patient, eliminated from Fig. 1 for the sake of clarity, showed only a moderate increase which occurred entirely within the normal

range (Fig. 2, *C*). The change that did occur, however, was significant (550 units per milliliter of packed cells), and represented a definite indication of red cell proliferation. This indication was subsequently confirmed by the increasing hemoglobin level.

In the 2 remaining patients, GOT levels were essentially stable, suggesting that if hemorrhage had occurred it was not associated with a marked marrow response. This was consistent with the absence of anemia (Fig. 2, *E* and *F*). In one, however (Fig. 2, *E*), there was a sharp drop in hemoglobin at the twenty-third day after the reported occurrence of a tarry stool. This drop was of short duration and was reflected in an only moderate increase in GOT. It is interesting that this increase in enzyme activity occurred some 12 days after the abrupt drop in hemoglobin level and presumable blood loss. Unfortunately, further studies on this patient could not be carried out.

In chronic hemorrhage, elevated enzyme levels are commonly seen throughout the period of anemia. Estimations of red cell GOT activity were carried out on

Table I. Red cell GOT activity in patients with chronic blood loss and anemia (Group B)

Patient No.	*Hemoglobin (Gm. per cent)*	*GOT activity (units/ml. packed cells)*
Group B-1: elevated GOT activity		
1	6.8	2,510
2	6.0	2,490
3	7.0	3,570
4	7.8	3,850
5	9.8	2,310
6	8.5	2,360
7	6.8	4,490
8	6.8	2,380
9	2.8	8,000
10	10.4	1,810
11	9.5	3,070
12	9.6	4,130
13	7.6	2,120
14	11.5	2,170
15	10.5	2,440
16	13.7	3,010
17	5.5	3,870
18	4.1	5,290
19	12.2	2,050
Group B-2: normal GOT activity		
20	7.9	890
21	11.7	1,520
22	9.8	750
23	8.0	1,510
24	10.4	1,020
25	7.0	1,020
26	10.0	1,000
27	12.4	1,050

Table II. Red cell GOT activity in patients with evidence of blood loss without anemia (Group C)

Patient No.	*Hemoglobin (Gm. per cent)*	*GOT activity (units/ml. packed cells)*
1	14.5	760
2	14.0	1,510
3	14.0	900
4	14.0	1,210
5	14.5	1,050
6	15.2	670
7	14.0	1,300

28 patients with gastrointestinal disease. The data, summarized in Table I, clearly demonstrate the marked increases that may be observed. More interesting, however, is the finding that 8 patients with anemia (Nos. 20 through 27) exhibited levels of activity within the normal range. Unfortunately, at the time these patients were seen, the history of blood loss was sufficient to prevent consideration of other explanations for the anemia, and further studies were not carried out. It would be of interest to determine whether additional studies would substantiate the hypothesis implicit in these findings, i.e., that a substantial number of the anemias found in association with gastrointestinal disease are more than simple manifestations of blood loss. The occurrence of nutritional disturbances would be not at all surprising.

The last 7 patients (Table II) showed clinical and radiologic evidence of gastric and duodenal ulcer with no anemia or history of melena. The normal GOT values seen in these patients provided objective confirmatory evidence that significant bleeding had not occurred within the preceding 30 to 45 days. Furthermore, these findings remove all doubt that gastrointestinal disease itself, in some unforeseen manner, does not precipitate the changes in transaminase activity encountered in those cases associated with chronic hemorrhage.

Discussion

The results presented above clearly indicate that, in otherwise normal patients, increased red cell proliferation secondary to acute blood loss is accompanied by definite increases in the GOT activity of the peripheral red cells. These findings offer further corroborative evidence of the adequacy of the view that increased enzyme activity is a function of decreased mean red cell age rather than cellular abnormality due to hematologic disease. It is quite probable that the extent of the increase is directly related to the degree of bone marrow response as well as to the extent of anemia brought about by a single episode of acute blood loss. In chronic hemorrhage, the picture no doubt is further complicated by continued blood loss and iron deficiency, as well as by a series of other unknown factors contributing to the effectiveness of the marrow response. Despite this, a rough inverse correlation between hemoglobin level and enzyme activity can be demonstrated (Fig. 3).

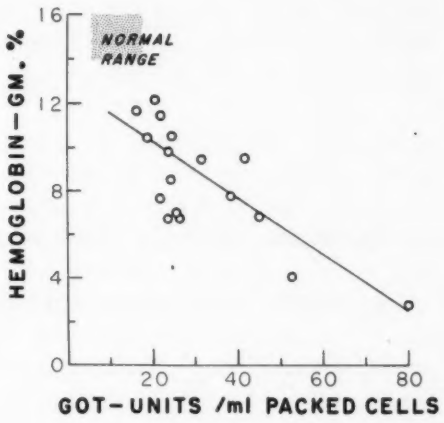


Fig. 3. Relationship between degree of anemia and red cell transaminase activity.

A more practical view of the data reveals that in the normal patient red cell transaminase activity remains constant within fairly narrow limits. Within 10 to 14 days after a bleeding episode, if it was severe enough to produce anemia, enzyme activity may increase from 50 to 200 per cent. These high levels may persist for some 30 to 40 days before returning to the normal range. These changes, though related to reticulocytosis, occur more consistently and over a more prolonged period of time. The data therefore suggest that red cell GOT activity can serve as an excellent parameter for evaluating the extent of blood loss. Since this parameter is not dependent on reticulocytosis, its usefulness extends beyond the limited period of reticulocytosis or even anemia.

With the exception of the 10 to 14 day lag period, similar changes can be found in patients with chronic blood loss anemia. In a certain considerable percentage of these cases, 30 per cent in this present limited study, normal GOT activities will be encountered. This has been interpreted as indicating an associated defect overlooked in the presence of a more easily recognizable explanation for anemia, i.e., chronic blood loss of an indeterminate severity. Further studies are being carried out to determine the adequacy of this interpretation.

REFERENCES

1. Sass, M. D., and Spear, P. W.: Whole Blood Transaminase Levels in Anemia, J. LAB. & CLIN. MED. **51**: 926, 1958.
2. Sass, M. D., and Spear, P. W.: Red Cell Transaminase Levels in Anemia. II. Thalassemia Minor, J. LAB. & CLIN. MED. **58**: 580, 1961.

Catalase activity and redox activity in platelets of patients with rheumatoid and nonrheumatoid inflammatory states

GRACE P. KERBY, M.D., and S. M. TAYLOR, B.A. *Durham, N. C.*

The catalase activity of human platelets and whole blood, as well as the redox activity of platelets, was compared in patients with and without inflammatory disease, including rheumatoid arthritis.

A slightly decreased level of catalase activity in whole blood of patients with rheumatoid arthritis was without significance ($p > 0.5$) on comparison with noninflammatory patients, and was of possible significance ($p < 0.5$) on comparison with nonrheumatoid inflammatory patients, after correction for erythrocyte level. Catalase activity per unit of platelets was the same in all groups. This did not support the likelihood of a general depression of enzymatic activity in platelets in rheumatoid or nonrheumatoid inflammatory states.

Slight decrease in basic redox activity of platelets from rheumatoid patients was noted in the absence of plasma (but not in 4 per cent plasma), and this persisted after addition of serotonin. Addition of serotonin in the presence of plasma markedly accelerated the in vitro redox activity of all platelets, although to a slightly lesser degree in platelets from rheumatoid than from those of nonrheumatoid patients. The findings suggested minor differences in the handling of serotonin by platelets from rheumatoid patients.

Recent studies in this laboratory[1] have shown a possibly significant decrease in vitro in the absorption of serotonin (5HT) by platelets in 40 per cent plasma from patients with rheumatoid and nonrheumatoid inflammatory states as compared to patients without inflammation. Subsequent studies of platelets in 4 per cent plasma have shown a correlation between the effect of added 5HT on redox activity of platelets and the ability of the platelets to take up 5HT.[2] We were interested in whether (1) the first observation reflected a general

From the Department of Medicine, Duke University Medical Center, Durham, N. C.
Supported by grants from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and the American Heart Association.
Received for publication March 22, 1960.

depression of enzymatic activity in platelets in inflammatory states, and (2) extension of the second observation to groups of patients would reveal a difference in the redox activity of platelets from rheumatoid and nonrheumatoid patients.

Since catalase has been reported recently[3] to be significantly decreased in the blood in rheumatic diseases and especially in rheumatoid arthritis (even after correction for the expected changes[4, 5] seen with variations in erythrocyte count), and since this enzyme was presumably unrelated to serotonin uptake, catalase activity of platelets was measured. No significant difference in catalase activity was found in the 3 groups of patients studied here as regards activity per unit of platelets. However, certain minor but consistent differences in redox activity of platelets were noted. Thus the results did not suggest over-all depression of enzymatic activity in platelets in inflammatory states but did suggest the probability of minor differences in the handling of 5HT by platelets from rheumatoid patients.

Materials and methods

Blood was drawn from hospitalized patients* by venipuncture, using siliconized syringes and dispensing as follows: About 9 ml. to each siliconized tube containing 1 ml. of 1 per cent disodium ethylenedinitrilotetraacetate (EDTA) in 0.7 per cent aqueous sodium chloride solution; about 2 ml. to a glass tube containing 5 mg. dry potassium oxalate and 7.5 mg. dry ammonium oxalate, which was placed at once in an ice bath (this sample was omitted in the redox studies); and a small sample for serum. Platelets were separated from the blood anticoagulated with EDTA by the method of Dillard and associates.[7]

A. Catalase studies. Platelets from 18 ml. of blood were washed once with saline, resuspended once to exactly 2.7 ml. in saline (at which time the number of erythrocytes per cubic millimeter of platelet suspension was counted and recorded), recentrifuged, and resuspended finally to exactly 2.7 ml. in demineralized water; a 0.5 ml. aliquot was used for biuret determination by the method of Jayle and co-workers,[8] and duplicate 1.0 ml. aliquots were used for determination of catalase activity as noted presently. The oxalated blood was kept iced until used for routine R.B.C. count and for determination of blood catalase activity in 1 ml. aliquots of 1:1,001 and 1:501 dilutions of the blood in iced saline. Catalase activity was determined by Jolles' thiosulfate method as modified by Sumner and Dounce.[9] Platelet catalase levels were accepted only if duplicate determinations checked within 5 per cent. Whole blood catalase levels determined on 2 different dilutions of blood were accepted only if checks within 7.5 per cent were obtained. A catalase unit was taken[10] as the amount of catalase required to give a calculated K_o value of 1, K_o being determined by the method of least squares.

B. Redox studies. Platelet buttons from 18 to 27 ml. of blood were carefully drained, and walls of tubes were wiped dry. Platelets were kept iced throughout preparatory procedures. They were resuspended to standard photometric density of 0.250 (O.D. 420 mμ on a Coleman Junior spectrophotometer, saline blank) in physiologic saline. Glucose, when added, was present in 0.0025 M concentration in the saline, and plasma was present in 10 per cent concentration. Dye reduction tests were then done as in previous studies,[2] using 2,6 dichloroindophenol (DIPH) as redox indicator dye, the final reaction mixture containing platelets (0.100 O.D. 420 mμ), balanced buffered salt solution (pH 7.4, phosphate 4×10^{-3} M), sodium cyanide (2×10^{-4} M), DIPH dye (10 μg per milliliter), and (as indicated in Figs. 1 and 2)

*As in previous investigations,[6] 3 groups of patients were studied, consisting essentially and as previously defined of Group I, with no evidence of inflammatory disease, Group II, with significant but nonrheumatoid inflammatory processes, and Group III, with unequivocal active rheumatoid arthritis.

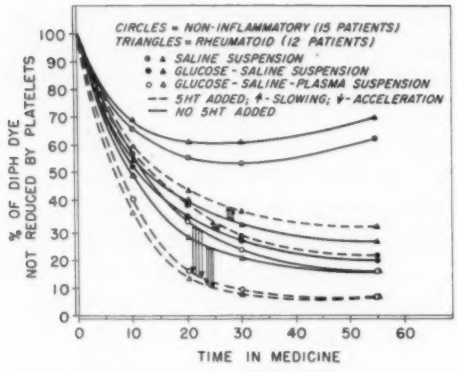

CIRCLES = NON-INFLAMMATORY (15 PATIENTS)
TRIANGLES = RHEUMATOID (12 PATIENTS)
SALINE SUSPENSION
GLUCOSE - SALINE SUSPENSION
GLUCOSE - SALINE - PLASMA SUSPENSION
5HT ADDED; ϕ - SLOWING; ϕ - ACCELERATION
NO 5HT ADDED
% OF DIPH DYE NOT REDUCED BY PLATELETS
TIME IN MEDICINE

Fig. 1. Comparison of redox activity of platelets from rheumatoid and non-rheumatoid patients; effect of added glucose, plasma, and serotonin *(5HT)* to balanced buffered salts in vitro.

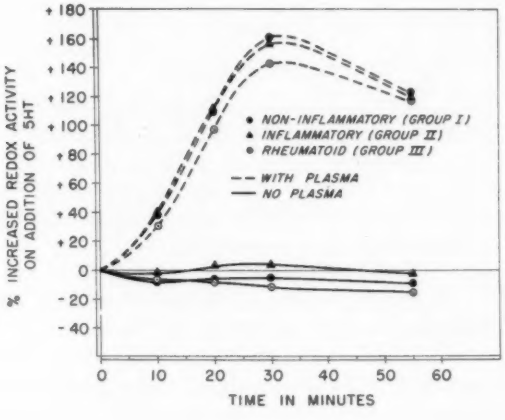


Fig. 2. Comparison of the effect of serotonin *(5HT)* on in vitro redox activity of platelets in various groups of patients, in the presence and absence of 4 per cent autologous plasma.

Table I

Group (No. of subjects)	*Catalase units* per 100 ml. whole blood*	*Catalase index†*	*Catalase units per 100 mg. platelet protein‡*
I. Noninflammatory (21)	18.84 ± 2.38 (S.D.)	3.87 ± 0.57	1.99 ± 1.41
II. Inflammatory (21)	19.14 ± 3.37	4.26 ± 0.73	1.62 ± 0.98
t, p values (vs. Group I)	*0.333, > 0.5*	*1.785, > 0.05*	*0.993, > 0.3*
III. Rheumatoid (15)	16.98 ± 2.98	3.80 ± 0.45	1.75 ± 1.32
t, p values (vs. Group I)	*2.042, < 0.05*	*0.413, > 0.5*	*0.511, > 0.5*
t, p values (vs. Group II)	*1.959, > 0.05*	*2.181, < 0.05*	*0.351, > 0.5*

*As defined in *Materials and methods.*

†Catalase units per 100 ml. whole blood per million R.B.C. per mm.³ blood.

‡After correction for erythrocyte contamination (made possible by determining erythrocytes per mm.³ of whole blood and of platelet suspension, and the catalase activity of the whole blood. The over-all catalase activity of the blood [due predominantly to erythrocytes] was taken as being an adequately accurate measure of erythrocytic catalase activity. Erythrocyte contamination of platelet suspensions was minimal but always present).

glucose ($1 - 10^{-3}$ M), plasma (4 per cent, with disodium EDTA in final 1.5×10^{-4} M), 5HT (as serotonin creatinine sulfate,* 5×10^{-4} M). C-reactive protein tests were done on serum from each patient.†

Results

Results of catalase studies are summarized in Table I; results of redox studies are summarized in Figs. 1 and 2.

A. Catalase studies. Although human platelets do exhibit catalase activity,[11] the bulk of blood catalase activity is recognized as being of erythrocytic origin. The observation reported by Kawakatsu[3] of significantly decreased amounts of blood catalase activity in rheumatic and rheumatoid states is at variance with observations on a few such patients reported earlier by Richardson and coworkers.[12] Schultz and Rose[13] found slight decreases in blood catalase activity more frequently in rheumatic fever than in other febrile states, but they did not regard this as a characteristic finding. In the present study a slight decrease was noted in blood catalase activity in rheumatoid patients; when corrected for number of erythrocytes present per unit of blood, this was without significance ($p > 0.5$) on comparison with noninflammatory patients, and of possible significance ($p < 0.05$) on comparison with nonrheumatoid inflammatory patients (Table I).

Platelets showed greater variation in catalase activity from individual to individual than did whole blood. No significant difference was noted between the rheumatoid and nonrheumatoid inflammatory groups on comparison with the noninflammatory group as regards catalase activity per unit of platelets. However, earlier studies[1] have shown‡ that blood from rheumatoid patients (and, to a lesser and insignificant degree, patients with nonrheumatoid inflammatory states) yields a larger amount of platelet protein per 100 ml. than does blood from patients with no evidence of inflammation. Thus the amount of catalase activity contributed by platelets to each unit of blood may be assumed to have been somewhat less in the patients without inflammatory states than in the rheumatoid group. This would seem to be probably without physiologic significance, since so little of the over-all blood catalase activity is derived from platelets in any event.

The physiologic role of catalase is not known; Keilin and Hartree[14] have presented experimental evidence for a function in coupled oxidations, but conclusive demonstration of such a physiologic role is lacking. The importance of the enzyme in any given cell or tissue must therefore remain a matter for speculation at present.

For purposes of the present study, the normal levels of catalase activity in platelets from patients with rheumatoid and nonrheumatoid inflammatory states do not suggest the likelihood of a general depression of enzymatic activity in such platelets.

*Abbott Laboratories, North Chicago, Ill.

†All of the patients in Group I had negative tests; all of those in Groups II and III had positive tests.

‡Groups comparable to those of the present study yielded milligrams of protein per platelets from 100 ml. blood as follows: (I) Noninflammatory, 34.5 ± 13.4 (S.D.); (II) inflammatory, 47.4 ± 21.5; (III) rheumatoid, 50.2 ± 12.6.

B. Redox studies. As noted in Fig. 1, rheumatoid platelets showed less redox activity than nonrheumatoid platelets in the absence of plasma. The difference was of some significance ($p < 0.05$ at 30 minutes, < 0.01 at 55 minutes). Nonrheumatoid inflammatory platelets showed a trend similar to that of the rheumatoid platelets but to a lesser degree ($p > 0.1$). When 5HT was added in the absence of plasma, the decreased redox activity in rheumatoid platelets persisted, whereas in nonrheumatoid inflammatory platelets the trend disappeared (Fig. 2). The decreased redox activity did not seem to reflect a general depression in enzymatic activity in the rheumatoid platelets, since catalase activity was unchanged.

In the presence of plasma, there was little significant difference in redox activity of platelets from the 3 groups of patients (Fig. 1). At 10 ($p < 0.01$) and 20 minutes ($p < 0.02$), redox activity of rheumatoid platelets exceeded that of nonrheumatoid platelets. When 5HT was added, this difference disappeared. Thus, while redox activity was markedly accelerated in all platelets on addition of 5HT in the presence of 4 per cent plasma, the degree of acceleration was slightly less in rheumatoid than in nonrheumatoid platelets (Fig. 2).

Discussion

The possible implications of these minor differences in handling of 5HT by rheumatoid platelets may lie in the realm of transport across cell membranes.[2] In earlier studies[1] platelets from both rheumatoid and nonrheumatoid patients with inflammatory states showed decreased capacity for absorption of 5HT under rigidly imposed conditions in vitro. Forty per cent plasma was used in those studies,[1] in contrast to the 4 per cent plasma which was found in later studies[2] to be optimal for demonstrating possible differences in this type of 5HT absorption in vitro by platelets. However, in the present study, the most sustained differences between rheumatoid and nonrheumatoid platelets were noted in the absence of plasma, where quite different mechanisms may be involved.[2] The differences noted in the presence of plasma were minor and permit only continued interest in investigation of the cell membrane in rheumatoid arthritis.

REFERENCES

1. Kerby, G. P., and Taylor, S. M.: In Vitro Absorption of Serotonin by Thrombocytes of Rheumatoid Arthritic and Non-Arthritic Individuals, Proc. Soc. Exper. Biol. & Med. 102: 45, 1959.
2. Kerby, G. P., and Taylor, S. M.: The Role of Chelation and of Human Plasma in the Uptake of Serotonin by Human Platelets, J. Clin. Invest. 40: 44, 1961.
3. Kawakatsu, C.: Blood Catalase Activity in Rheumatic Diseases, Nippon Onsen Kiko Gakkai Zasshi 22: 246, 1958 (CA 53: 5478c, 1959).
4. Krumbhaar, E. B., and Musser, J. H.: The Catalase Content of the Blood in Different Types of Anemia, J. A. M. A. 75: 104, 1920.
5. Beutler, E., and Blaisdell, R. K.: Iron Enzymes in Iron Deficiency. II. Catalase in Human Erythrocytes, J. Clin. Invest. 37: 833, 1958.
6. Kerby, G. P.: The Effect of Inflammation on the Hexuronate-Containing Polysaccharides of Human Plasma, J. Clin. Invest. 37: 962, 1958.
7. Dillard, G. H. L., Brecher, G., and Cronkite, E. P.: Separation, Concentration, and Transfusion of Platelets, Proc. Soc. Exper. Biol. & Med. 78: 796, 1951.

MATHEWS LIBRARY
A.S.U. TEMPE, ARIZ.

8. Jayle, M. F., Boussier, G., and Badin, J.: Microdosage des proteins seriques par la méthod du biuret, Bull. Soc. chim. biol. (Paris) **33**: 881, 1951.
9. Sumner, J. B., and Dounce, A. L.: Methods in Enzymology, vol. 2, New York, 1955, Academic Press Inc., p. 780.
10. Tria, E.: Anticatalase, J. Biol. Chem. **129**: 377, 1939.
11. Iglauer, K., and Weber, S.: Die Katalasewirkung der Thrombocyten, Biochem. Ztschr. **234**: 489, 1931.
12. Richardson, M., Huddleson, I. F., Trustdorf, M., and Bethea, R.: Study of Catalase in Erythrocytes and Bacteria. III. Catalase Activity of Erythrocytes From Humans and Animals in Various Pathological States, Arch. Biochem. **47**: 388, 1953.
13. Schultz, M. P., and Rose, E. J.: The Catalytic Potency of the Blood in Rheumatic Fever, United States Public Health Reports **54**: 343, 1939.
14. Keilin, D., and Hartree, E. F.: Properties of Azide-Catalase, Biochem. J. **39**: 148, 1945.

The use of I^{131}-labeled contrast media in scintillation scanning of the kidney

THOMAS P. HAYNIE, M.D., MOHAMMED M. NOFAL, M.D., EDWARD A. CARR, JR., M.D., and WILLIAM H. BEIERWALTES, M.D.
Ann Arbor, Mich.

Various I^{131}-labeled contrast media were injected intravenously into 10 dogs in 60 experiments to evaluate factors that would aid in the production of a radioactive renal scintigram with the best contrast and definition. The contrast media used, the dose of radioactivity, the rate of administration of the dose, the time of starting the scan, and the type of collimator used were of importance. The state of dehydration, the use of adjunctive drugs, an abdominal binder, and carrier did not enhance the quality of the scintigram. By using 150 to 200 μc of I^{131}-labeled ortho-iodohippuric acid, administered by intravenous infusion at the rate of 2 to 4 μc per minute, starting the scan 20 to 30 minutes after the start of the infusion, using a 3 by 2 inches scintillation crystal detector, with a 19 hole collimator, a spectrometer, and photoscanning, it was possible to delineate areas of infarction in dog kidneys scanned before and after ligation of renal arteries. Human kidneys also were delineated with this technique.

The visualization of internal organs by radioistope scanning has proved a useful clinical procedure in the evaluation of disorders of the thyroid gland, and, in some instances, of the liver and brain. Wagner and associates,[1] in an article on scintillation scanning, mentioned the use of Hg^{203}-labeled chlormero drin for visualizing the kidney. Denneberg and Hedenskog[2] published one scintigram of the kidneys with I^{131}-labeled Diodrast (the diethenalamine salt of 1,4 dehydro-3,5-diiodo-4-oxopyrid-1-glacetic acid) and Hypaque (sodium 3,5-diacetamido-2,4,6-triiodobenzoate). No details of technique were given.

This report presents the development of a technique for radioisotope scanning of the kidneys and an evaluation of the ability of this technique to detect infarction of part of the kidney.

From the Departments of Internal Medicine and Pharmacology, and the Clinical Radioisotope Unit, University of Michigan Medical Center, Ann Arbor, Mich.
Supported by a grant from the United States Atomic Energy Commission, Project 15.
Presented at the Midwestern Section Meeting of the American Federation for Clinical Research, November 3, 1960, in Chicago, Ill.
Received for publication Jan. 30, 1961.

Materials and methods

Outline of studies. A basic schema for scintiscanning the kidneys was first devised. Then various factors were changed and the effect on the quality of the scintigram observed. After the optimum delineation of the normal renal outline was obtained, a standard procedure was adopted to study the effect of renal vascular occlusions on the renal scintigram.

Basic schema. In the study of important variables in the quality of the scan, the equipment consisted of a collimated scintillation probe with a 1 by 1 inch sodium iodide crystal with a 6 hole collimator, a pulse height selector, and a direct recording stylus.* In later experiments on the effect of renal infarction, photoscanning equipment† with a 3 by 2 inches sodium iodide crystal with a 19 hole collimator was also employed. Female dogs, weighing 10 to 12 kilograms, were anesthetized and placed in the prone position with sandbags under the abdomen. I^{131}-labeled contrast media was administered by vein into the forelimb, and the area between the tenth dorsal and fifth lumbar vertebrae was scanned from one side of the body to the other.

A total of 60 experiments were performed in 10 dogs. One dog was sacrificed 20 minutes after administration of the labeled contrast media and the excised kidneys, immersed in water, were scanned repeatedly under varying conditions of geometry.

Important variables. The following factors were found to be of most importance in obtaining renal scintigrams with optimum contrast and definition:

1. CONTRAST MEDIA. I^{131}-labeled Diodrast, Renografin (diatrizoate methylglucamine), and Hippuran (ortho-iodohippuric acid) were evaluated. Hippuran seemed superior, although a clear difference between Hippuran and Renografin was not established in these experiments. Diodrast concentrated in the liver but, when preceded by a dose of stable carrier, also gave satisfactory scans.

2. DOSE. Amounts of labeled contrast media containing from 100 to 500 μc of I^{131} were employed. The minimal dose permitting satisfactory scanning was found to be between 150 and 200 μc.

3. RATE OF ADMINISTRATION. Using rate meter, rectilinear recorders, and I^{131}-labeled Renografin, the concentration of radioactivity in the kidney and extrarenal "background" tissues, as represented by kidney and head counts, were compared following a rapid single injection of 200 μc, a single injection of 100 μc followed by a slow intravenous infusion of 100 μc (4 μc per minute), and a slow intravenous infusion of 200 μc (4 μc per minute). The maximum contrast ratio of kidney to "background" tissues was obtained with the slow intravenous infusion and was about 4 to 1. The maximum activity in the kidney was reached in about 30 minutes after beginning the infusion, while the maximum activity in the blood was reached in about 10 minutes.

4. SCANNING APPARATUS. Using a scanner with a 1 by 1 inch crystal and a recording stylus, a 1 hole and a 6 hole collimator (focusing at 2 inches) were compared. The 6 hole collimator, positioned at skin surface, gave the best scan with this equipment. Later, a photoscanner with a 3 by 2 inches crystal and a 19 hole collimator was compared with the aforementioned scanning apparatus. The use of the photorecorder gave definitely superior scanning images. Attempts to use higher (31 hole) collimation with the photorecorder resulted in a marked reduction in quality of the scan at this level of radioactivity.

Variables of little or no importance under the conditions studied. A number of ancillary measures also were evaluated in an effort to improve the quality of the scans, but were for the most part ineffective.

1. DEHYDRATION. The dogs were deprived of liquid for 24 hours before the scan but no improvement in the scan was noted.

2. ADJUNCTIVE DRUGS. One half gram of probenecid (Benemid) was given *orally* each day for 3 days prior to the scan in an effort to slow renal excretion. In another experiment 20

*Nuclear-Chicago Corporation, Des Plaines, Ill.
†Picker Magnascanner, Picker X-Ray Corporation, White Plains, N. Y.

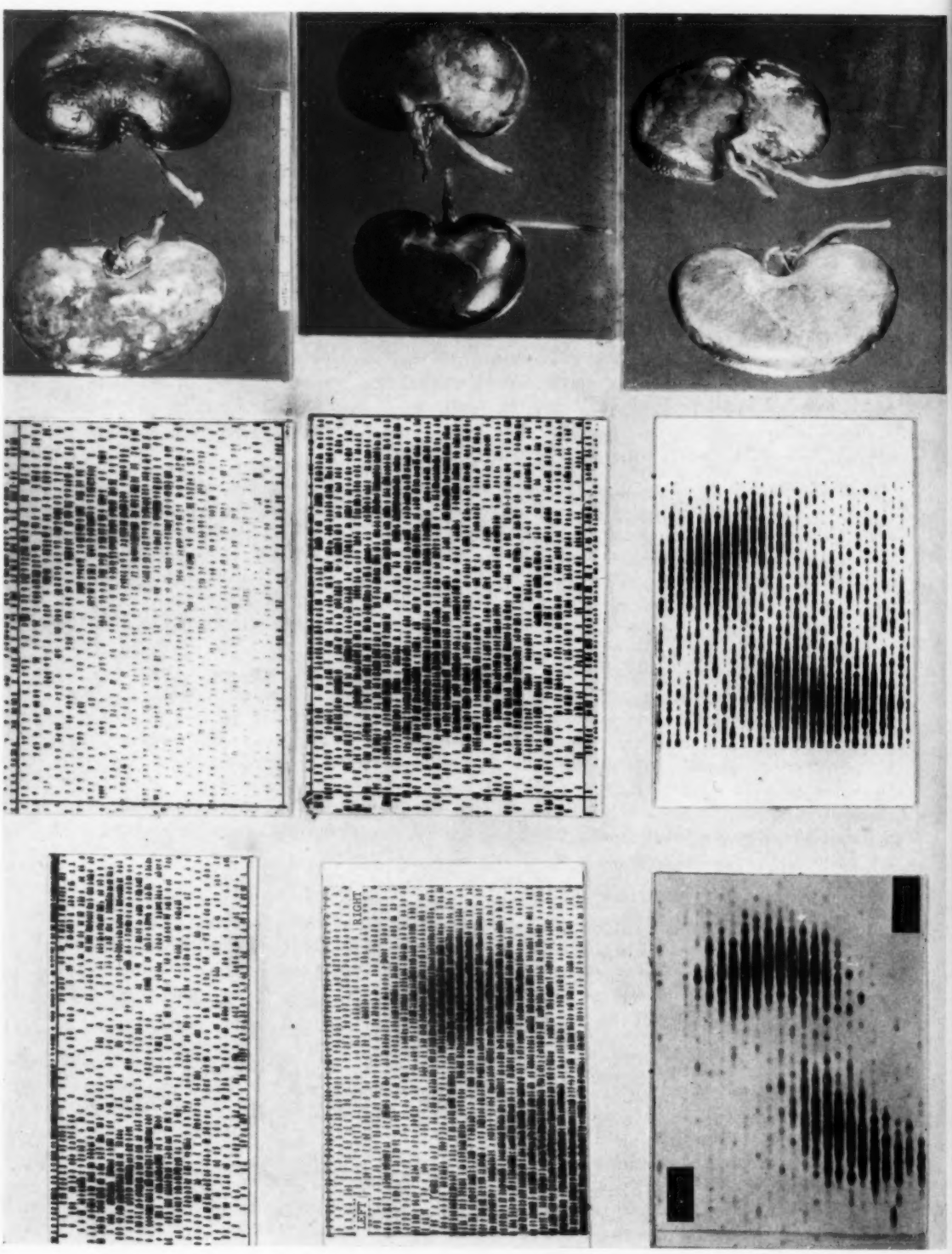


FIG. 1-A. For legend see opposite page.

Fig. 1A. For legend see opposite page.

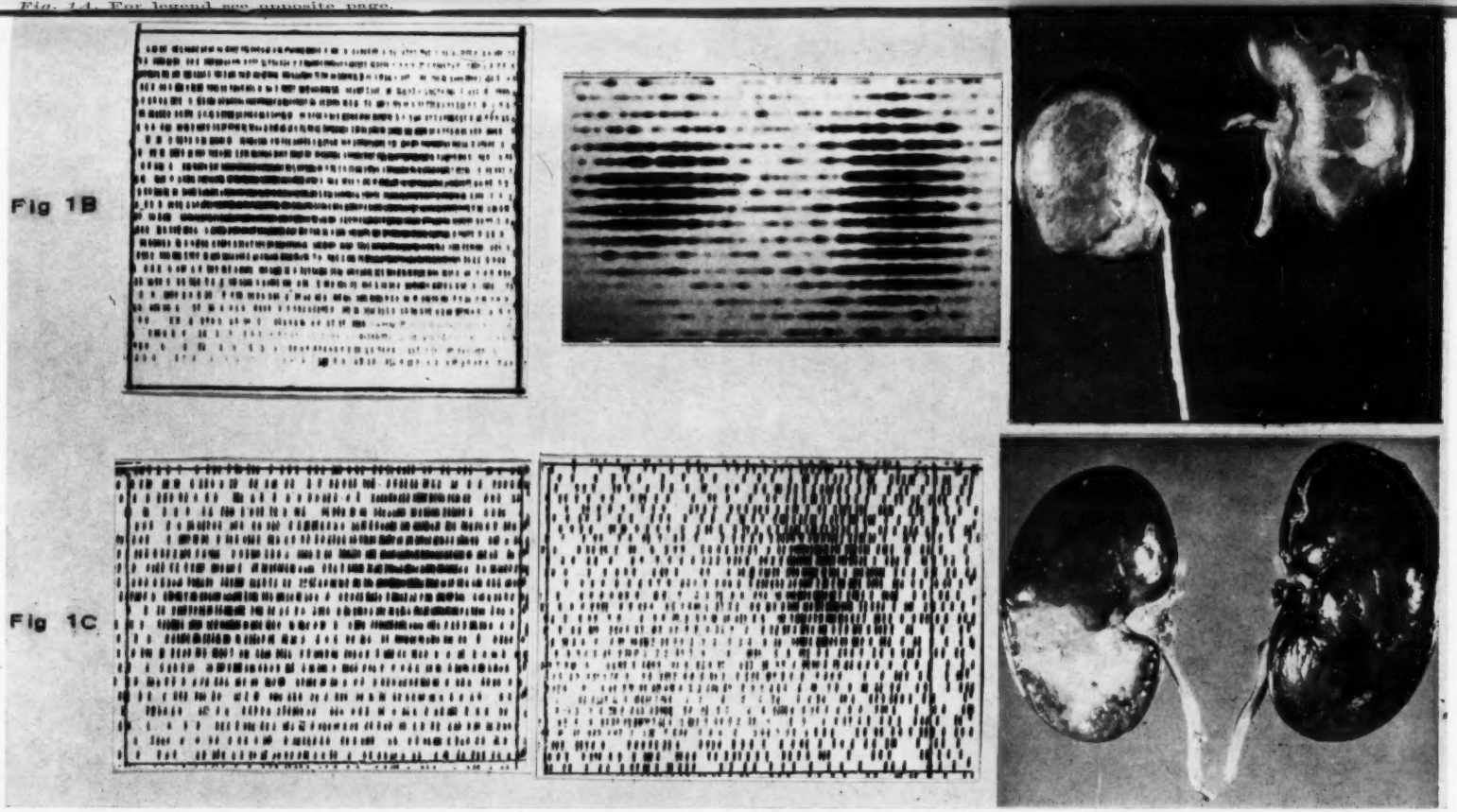


Fig. 1A. Pre- and postoperative renal scintigrams in 3 dogs which had renal artery ligations after the first scintigram. In each pair of kidneys, the right kidney is on the reader's right. The infarcts visualized by the pathologist at sacrifice are presented at the far right. Illustrations are as follows, reading left to right: Preoperative scintigram, postoperative scintigram, and gross pathology. TOP. Dog No. 1, third postoperative day. Operation performed: Ligation of all branches of left renal artery. MIDDLE, Dog No. 2, third postoperative day. Operation performed: Ligation of one branch lower right renal artery. BOTTOM, Dog No. 3, twenty-fifth postoperative day. Operation performed: Ligation of one branch lower right renal artery. *Figs. 1B* and *1C*. Postoperative scintigrams in 2 dogs comparing photorecording and dot recording in the same animal (Fig. 1*B*) and demonstrating a progressive decrease in uptake after operation (Fig. 1*C*). Illustrations are as follows, reading left to right: TOP, Dog No. 4, eighteenth postoperative day: Postoperative scintigram, dot recorder; postoperative scintigram, photorecorder; and gross pathology. Operation performed: Ligation of both branches lower left renal artery. BOTTOM, Dog No. 5, first postoperative scintigram (seventeenth postoperative day); second postoperative scintigram (thirty-second postoperative day); and gross pathology. Operation performed: Ligation of one branch lower left renal artery.

Table I. Standard technique. Factors found to be ideal with 2 different types of recording equipment in producing a scintigram with maximum contrast and definition in the dog and human

Dose
I^{131}-labeled Hippuran
150 to 200 μc diluted in 150 to 250 ml. 5% dextrose in water
Rate
Administer intravenously at rate of 2 to 4 μc per minute
Equipment
A. Direct recording stylus, 1 by 1 inch crystal, 6 hole collimator
B. Photoscanner, 3 by 3 inches crystal, 19 hole collimator
Time interval
Interval between beginning infusion and beginning scan: 20 to 30 minutes
Duration of scan: 30 to 40 minutes

units of aqueous vasopressin (Pitressin) was injected intramuscularly, 1 hour before beginning the administration of the labeled material, in an attempt to concentrate the urine. Neither measure improved the quality of the scan.

3. CARRIER. The administration of 0.5 Gm. of stable iodopyracet (Diodrast) intravenously at the time of the infusion blocked the uptake of I^{131}-labeled Diodrast by the liver, but the renal scans were not superior to those obtained with "carrier free" I^{131}-labeled Hippuran. In another experiment the administration of 0.5 Gm. of stable Hippuran with the infusion of I^{131}-labeled Hippuran also resulted in no improvement.

4. ABDOMINAL CONSTRICTION. An abdominal girdle applied to the lower abdomen to retard ureteral flow resulted in no significant improvement in the scan.

Standard technique. As a result of this experimentation we derived a standard technique (Table I) for scintillation scanning of the kidney which was then used in our investigation of experimental renal vascular lesions in dogs.

Effect of experimental renal vascular lesions. Following the performance of a renal scintiscan, an exploratory laparotomy was performed through an anterior abdominal incision, at which time the status of the 2 kidneys was ascertained and the arterial supply of one of the kidneys defined. Ligations of various renal arteries were then carried out. Postoperative

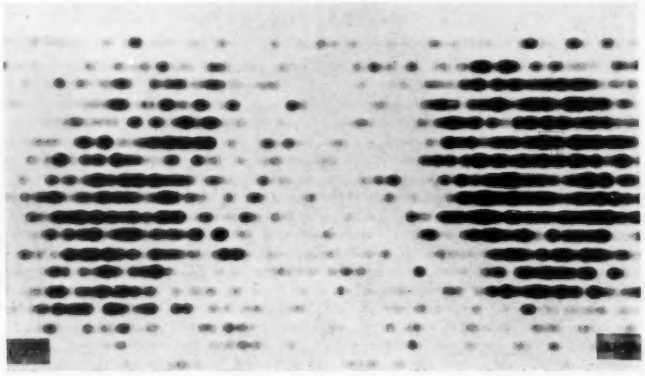


Fig. 2. Photoscintigram of the kidneys of a 54-year-old man with hypertension. At operation he was found to have bilateral renal vascular disease, predominantly in the left kidney.

scintiscans were performed at intervals of 3 days to 1 month postoperatively; the dogs were then sacrificed and the kidneys were removed and examined grossly and microscopically. The results of these investigations in 5 experimental animals are summarized in Fig. 1.

In these experiments we have evaluated the effects of several patterns of renal infarction produced by ligating renal arteries or their branches and have compared the effects of acute lesions as well as long-standing infarcts on the renal scans.

Results

All of the lesions produced changes in the postoperative scintiscan when compared with the preoperative scan. The change consisted of a reduction in concentration of radioactivity over the area of the infarction, when the renal outline (determined by the preoperative scan and confirmed at autopsy) was superimposed on the postoperative scintiscan. This qualitative difference was quantitated in the last 2 experiments by rate meter counting, which demonstrated a three- to fourfold decrease in radioactivity in the infarcted area as compared to counts over noninfarcted areas of the same and the contralateral kidney. The infarcts in these experiments varied in size from 6 by 3 cm. to 3 by 2 cm. Histopathologically, they revealed recent infarction with hemorrhage and necrosis in the first week, and organizing infarcts with marked tubular atrophy at 1 month postoperatively. The unaffected renal parenchyma was not altered either grossly or microscopically and the animals appeared to be healthy. Intra-arterial blood pressure readings performed preoperatively and 1 week to 1 month postoperatively in 2 dogs showed no significant change.

Three experiments, presented in Fig. 1*A*, show the effects of experimentally produced renal infarction on the postoperative scintigram. In each case a preoperative scintigram had revealed a normal renal outline. The direct recording stylus was employed in Dogs Nos. 1 and 2, and photoscanning in Dog No. 3. In Fig. 1*B*, postoperative scintigrams in the same dog performed with photo- and dot recording are compared. In Dog No. 5 two postoperative scans revealed progressive decrease in uptake in the operated kidney, over 1 month's time.

Encouraged by our experiments in animals, we have begun to perform renal scintigrams in humans, using the same technique as in the experimental animal. We have obtained good kidney outlines in the human, as shown in Fig. 2, and are at present conducting a clinical trial to compare the renal scan with the underlying pathology found at operation or at autopsy.

Discussion

This work suggests that the technique described gives sufficiently good delineation of the kidney to permit reasonably accurate scanning. The potential clinical usefulness of the renal scintigram is also suggested by the results presented in dogs in which specific vascular lesions have been created.

In considering the applicability of the present method to humans, 4 points should be noted: (1) The biologic half-life of the labeled compounds used in this experiment is shorter than that of many isotopic procedures in current routine use. Indeed, it is the rapid renal excretion of these compounds which have made them available for renal diagnostic studies. The calculated whole body

dose is less than 10 millirads. (2) A very small amount of contrast media is administered; this may reduce the danger of untoward immediate reactions in iodine-sensitive patients. Although such reactions are not common in conventional excretory urography, they have on occasion proved fatal. (3) Our studies have been performed with presently available isotopes and equipment in which one reasonably may expect further improvements. An isotope of iodine with shorter half-life or less beta radiation, such as I^{125}, may prove to be of value. How small a lesion can be detected also must be further determined, and the effectiveness of the I^{131} technique should be compared with other isotope techniques, such as the use of Hg^{203}-labeled compounds. (4) The demonstration of frank renal infarctions is of importance in respect to possible sources of hypertension, but even more important is the possible effect of local ischemia, without infarction, on the scan. This effect remains to be determined.

REFERENCES

1. Wagner, H. N., Jr., McAfee, J. G., and Mozley, J. M.: Medical Radioisotope Scanning, J. A. M. A. **174**: 162, 1960.
2. Denneberg, T., and Hedenskog, I.: The Radioactive Hypaque Renogram: Assessment of Renal Function With Automatic External Scintillation Detection Equipment, Acta med. scandinav. **165**: 61, 1959.

The comparison of the apparent volume of distribution of large molecular weight dextran and Evans blue dye

WALTER LYON BLOOM, M.D.* *Atlanta, Ga.*

In hospitalized patients a simultaneous measurement of the apparent volume of distribution of Evans blue dye was compared with that of 3 different molecular size ranges of dextran. Statistical analysis of relationship of the slope of dye or dextran concentration with time indicated that extrapolation to zero time increased the variability of the data. Simultaneous measurement of the apparent volume of distribution of dye and dextran in the same patient indicated small differences in the 2 methods. Large molecular weight dextran fractions thus may be used to measure plasma volume. The dextran measured in whole blood was shown to provide a means of estimation of whole blood volume. A chemical estimate of hematocrit was also demonstrated. The use of macromolecules of defined molecular range have been shown to present new possibilities of estimation of vascular volumes as well as providing an eventual method for chemical measurement of capillary permeabilities.

Plasma volume has been estimated by the determination of apparent volume of distribution (AVD) of Evans blue dye (T 1824) which is protein bound[1] and by radioactive iodine bound to albumin. Both of these methods are in good agreement.[2] Although the methods have been generally accepted, they are not without limitation. Measurement of the volume of distribution of dyes is of questionable value in the presence of lipemia, hemolysis, or serum pigmentation, and repeated studies may produce temporary skin pigmentation. The use of radioactive materials has limitations for repeated measurements and requires complicated and costly equipment. Furthermore, the space measured is a function of the weight and shape of the albumin molecule, whereas the space occupied by molecules of

From The Research Laboratories, Piedmont Hospital, Atlanta, Ga.
Supported by a United States Army grant, Contract No. DA-49-007-MD-932.
Received for publication May 21, 1959.
*Director of Medical Education and Research, Piedmont Hospital, Atlanta, Ga.

different weight and shape might not be the same as albumin. The basic principle of these methods depends upon estimating the dilution of tagged macromolecules which remain in the circulation. Large molecular weight dextran presented advantages as a substance which could be used for this type of measurement, since the material can be fractionated in different molecular ranges, can be measured chemically, is independent of the metabolic changes which influence serum proteins, and can be measured as easily in whole blood as in plasma. Furthermore, the molecular size of the fractions used in this study were larger than albumin and leakage from the circulation might be decreased. Recently, 2 studies have been published on the apparent volume of distribution of a single fraction of large molecular weight and the comparison of the dextran space to radioactive iodine–tagged albumin volume of distribution.[3, 4]

The results to be presented showed that the AVD of 3 ranges of dextran fractions was similar to the Evans blue dye space. The whole blood volume (WBV) calculated from the T 1824 space and the hematocrit was not essentially different from the AVD of dextran in whole blood. The observed venous hematocrit was not significantly different from the chemical hematocrit determined from the ratio of distribution of dextran in the whole blood and plasma.

Materials and methods

During this investigation, a simultaneous comparison of the apparent volume of distribution of dye protein and polysaccharide macromolecules was measured in 35 male and female hospitalized patients. The patients ingested a supper low in fat and then fasted 12 to 18 hours preceding the measurement of plasma volume. Three different sizes of dextran molecules were studied: National Research Council (NRC) 6, 7, and 8. The molecular range of these fractions is presented in Table I. These fractions were studied in different groups of patients, i.e., NRC 6 in 16 patients; NRC 7 in 12 patients; and NRC 8 in 7 patients. Fifty milliliter volumes of the 6 per cent dextran fraction were placed in 150 ml. infusion bottles and autoclaved. Since the dextran used was isosmotic, no extravascular fluid would be drawn into the circulation. This unit was then connected with a venous infusion apparatus described by Schultz and associates.[2] The dye (T 1824) and the dextran were administered simultaneously. The dextran container was flushed with approximately 25 ml. of saline to assure entry of all of the measured dose into the circulation. Blood samples were taken at 20, 40, and 60 minutes after injection. Dye concentration in the plasma was measured as described by Gregerson and Rawson.[5] Dextran was determined on whole blood by the method of Bloom and Willcox[6] and on plasma modified by the use of trichloroacetic acid protein-free filtrates. This technique reduced the blank determinations to 2 mg. per cent. Hematocrits were determined by the method of Wintrobe on the control blood and on each of the 3 samples obtained for dye and dextran estimates.

Table I. Molecular weight of National Research Council dextran fractions*

Fraction	*Average*	*Low*	*High*
NRC 6	194,900	129,000 (4.8%)	232,500 (7.4%)
NRC 7	255,000	200,000†	300,000
NRC 8	412,000	300,000†	500,000

*These specifications were supplied by the National Research Council.

†High and low 10 per cent fractions were not made on these preparations because of difficulty of narrow fractionation at higher molecular weights.

Calculations were based on the average apparent volume of distribution for 3 samples as described by the following:

$$\text{AVD dextran or T 1824} = \frac{\text{milligram injection}}{\text{mean plasma concentration in milligram}} \times 100.$$

The dextran whole blood volume was similarly calculated by the mean whole blood dextran concentration. The calculated whole blood volume was derived as follows:

$$\frac{\text{AVD T 1824} \times 100}{100 - \text{observed venous hematocrit.}}$$

The chemical hematocrit was determined by the following formula:

$$1 - \frac{\text{dextran concentration in milligram whole blood}}{\text{dextran concentration in milligram plasma}} \times 100.$$

The data taken as a whole suggest some tendency for concentrations of the dye T 1824 and of dextran in plasma (but not hematocrits or dextran concentrations in whole blood) to fall with time. However, the composite regressions (Table II) in the former graphs (calculated on groups of 6 to 16 subjects) are all small and generally of only borderline significance: in 4 series, $p = < .05$; in 1 case, $p = < .02$; and in 1 other case, $p = < .01$. The individual slopes compared with the variances from regression are not significant in any case (i.e., no significance may be attached to any one departure from zero slope) (Table III). Hence, the relationship to time might be used profitably only in connection with averages from groups of subjects, and attempts to extrapolate to zero time in individual subjects would most probably only increase the variability of the data. For comparisons of observations made on the same individuals by different methods, the averages of the triplicate observations, considered without regard to time, are probably the best estimates to use.

Table II. Composite regressions* of analysis of dextran and T 1824 concentrations and hematocrit in triplicate determinations at 20 minute intervals

	Mean	*Regression coefficient*	sB_c†	*Student T*	*Degrees of freedom*	*Probability*
NRC 6						
Observed hematocrit	29.59	+0.0077	0.0064	1.197	31	< .2
T 1824	461.94	−0.3384	0.187	1.81	31	< .05
Plasma dextran	105.31	−0.0702	0.040	1.755	31	< .05
NRC 7						
Observed hematocrit	29.06	− .00125	0.0081	0.154	23	< .8
Dextran hematocrit	27.89	+ .0629	0.084	0.749	11	< .4
T 1824	439.26	− .592	0.250	2.368	23	< .02
Plasma dextran	110.50	− .0781	0.0325	2.404	23	< .02
Whole blood dextran	80.58	− .0848	0.099	0.856	11	< .4
NRC 8						
Observed hematocrit	33.2	+ .0114	.0071	1.61	13	< .1
Dextran hematocrit	31.85	− .0682	.038	1.795	13	< .1
T 1824	410.4	− .3136	.158	1.985	13	< .05
Plasma dextran	97.66	− .1046	.0366	2.86	13	< .01
Whole blood dextran	65.39	+ .0107	.0383	0.279	13	< .7

*Snedecor, George W.: Statistical Methods, ed. 5, Iowa City, 1959, Iowa State College Press.

†sB_c = Standard deviation of the regression coefficient.

Table III. Example of analysis of variance*: plasma dextran NRC 8 (most significant slope) (7 series; b = –0.1046 [composite[b] = average[b] in these cases])

Variance	*Sum of squares*	*Degrees of freedom*	*Mean square*	*F*
A. Within subjects	158.6	14	(11.3)	
B. Composite slope	61.3	1	61.3	8.2†
C. Variance from composite slope	97.3	13	7.48	
A. Within subjects	158.6	14		
D. Individual slopes, total	114.6	7	16.4	2.61 (n.s.)
E. Variance from individual slopes	44.0	7	6.29	
F. Differences among slopes (c–e = 97.3 – 44.0)	53.3	6	8.9	1.4 (n.s.)

Average variance of individual regressions:
$Vbi = Vri/SX^2 = 6.29/800 = .00788$; average $S.E.b_i = .083$
$Vb_c = Vc/S(SX^2) = 7.48/5600 = .00134$; $S.E.b_c = .0316$.

*Snedecor, George W.: Statistical Methods, ed. 5, Iowa City, 1959, Iowa State College Press.
†p = .02 – .01.

Table IV. Comparison of the apparent volume of distribution of T 1824 and NRC dextran fractions

T 1824 (ml.)	*NRC 6 (ml.)*	*Δ (ml.)*	*T 1824 (ml.)*	*NRC 7 (ml.)*	*Δ (ml.)*	*T 1824 (ml.)*	*NRC 8 (ml.)*	*Δ (ml.)*
3,712	3,397	–315	2,626	2,636	+ 10	2,637	2,969	+ 332
3,351	3,121	–430	2,642	2,585	– 57	3,085	2,850	– 235
4.739	3,817	–922	6,203	4,841	–1,362	3,921	4,599	+1,034
3,502	3,460	– 42	3,796	3,864	+ 68	2,888	2,720	– 168
3,778	3,754	– 24	2,949	3,116	+ 167	3,989	2,994	– 995
3,719	3,090	–629	3,591	3,194	– 397	4,998	4,746	– 252
3,284	3,597	+313	3,821	4,040	+ 219	3,633	3,738	+ 105
3,517	2,658	–859	3,179	3,292	+ 113	$\overline{X} = -26$		
2,693	3,212	+519	5,007	4,216	– 791	*S.E.* = 234.8		
2,796	2,699	– 79	2,290	1,852	– 438	$p = < .3$		
2,362	2,420	+ 58	3,461	2,974	– 487			
3,214	2,635	–579	3,032	2,281	– 751			
5,336	4.774	–562	$\overline{X} = -309$					
2,325	2,283	– 42	*S.E.* = 140.1					
2,677	2,424	–253	$p = < .05$					
2,543	2,855	+312						
$\overline{X} = -222$								
S.E. = 105.6								
$p = < .05$								

Results

Simultaneous determinations of 2 apparent volumes of distribution on the same patient made it possible to compare the difference of measurement. Statistical analysis of the differences of dextran and dye in plasma indicated that the AVD of dextran fractions 6 and 7 was smaller than the dye space. The

differences were of borderline significance ($p = < 0.05$) (Table IV). The AVD of dextran NRC 8 was the same as that of T 1824.

Analysis of the linear regression of the T 1824 volumes on the dextran volumes shows a significant relationship between dye and dextran measurements for each fraction as well as the pooled data (Table VII). Fig. 1 presents the linear regression plotted for the volumes determined for the 3 dextran fractions plotted against the data obtained by the T 1824 method.

Whole blood volume was calculated from the plasma volume determined by the dye T 1824 and the hematocrit and compared with the AVD of dextran in the whole blood. Whole blood volume determined from dextran space did not significantly differ from the calculated whole blood volume (Table V). Although the whole blood AVD of dextran NRC 7 is suggestively smaller than the whole blood volume calculated from the dye space and the hematocrit, the difference is not significant.

The linear regression of blood volumes determined by both methods shows

Table V. Comparison of the apparent volume of distribution (in milliliters) of dextran in whole blood and the whole blood volume calculated from T 1824 space and venous hematocrit

Calculated T 1824 (ml.)	*NRC 7 (ml.)*	*Δ (ml.)*	*Calculated T 1824 (ml.)*	*NRC 8 (ml.)*	*Δ (ml.)*
5,798	5,683	− 115	3,860	4,193	+ 333
3,789	4,278	+ 489	5,508	5,085	− 432
6,362	5,818	− 544	5,569	5,767	+ 196
3,544	2,488	−1,056	4,491	4,449	− 42
5,220	4,060	−1,160	6,985	4,895	−2,090
4,805	3,555	−1,250	7,482	7,166	− 316
			4,294	4,529	+ 235
	$\overline{X} = -606$			$\overline{X} = -302$	
	S.E. $= 280.9$			*S.E.* $= 316.5$	
	$p = < .05$			$p = < .3$	

Table VI. Comparison of 1 minus the ratio of dextran in whole blood and plasma with the observed venous hematocrit

Observed	*NRC 7*	*Δ*	*Observed*	*NRC 8*	*Δ*
34.1	28.9	− 5.2	31.7	29.2	−2.5
16.1	23.0	+ 6.9	44.0	44.0	0
21.3	27.5	+ 6.2	29.6	20.2	−9.4
35.6	25.6	−10.0	35.7	38.9	+3.2
33.7	26.6	− 6.9	42.9	38.8	−4.1
36.9	35.9	− 1.0	33.2	33.8	+0.6
			15.4	17.5	+2.1
	$\overline{X} = 1.67$			$\overline{X} = -1.44$	
	S.E. $= 2.20$			*S.E.* $= 1.63$	
	$p = < .4$			$p = < .4$	

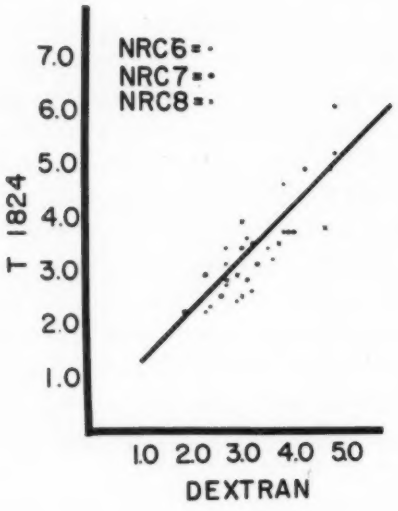


Fig. 1. Linear regression of each dextran determination plotted against the simultaneous T 1824 determination. Results plotted in liters of apparent volume of distribution of T1824 dye or dextran.

a significant relationship between the 2 measurements for each fraction as well as the pooled data (Table VII and Fig. 1).

Since dextran NRC 7 and 8 were determined in both the whole blood and the plasma, it was possible to derive the chemical hematocrit and compare it with the observed venous hematocrit measured by the centrifugal method. It was shown in the presentation of method that there was no significant change in venous or chemical hematocrits drawn at 20 minute intervals (Table II). This permitted the use of an average hematocrit derived from the 3 determinations. Analysis of the differences in the centrifugal and chemical dextran (NRC 7 and 8) determinations of the hematocrit showed both methods produced the same results (Table VI).

Table VII. Linear regressions of T 1824 and dextran volume of plasma distribution

Fraction	*Mean T 1824 plasma (Volume/L.)*	*Mean dextran plasma (Volume/L.)*	*Coefficient of regression*	*Deviation*	*Student T*	*Degrees of freedom*	*Probability*
NRC 6	3.15	3.13	1.06	.364	2.82	14	$< .025$
NRC 7	3.55	3.25	1.24	.328	3.78	10	$< .005$
NRC 8	3.60	3.53	.76	.23	3.3	5	$< .025$
Pooled data	3.46	3.25	1.02	.134	7.6	33	.001
	T 1824 whole blood volume	Dextran whole blood volume					
NRC 7	4.92	4.30	.697	2.9	3.18	4	$< .05$
NRC 8	6.36	6.01	1.06	.360	2.9	5	$< .05$
Pooled data	4.92	4.76	.825	.178	4.6	11	.001

Discussion

The apparent volume of distribution of 3 large molecular weight dextran fractions has been shown to be similar to the AVD of the dye T 1824. Our results are similar to those recently reported on a comparison of large molecular weight dextran and radioactive iodine–tagged albumin volumes of distribution.[4] Since the molecular size of the 3 dextran fractions is larger than the albumin which is tagged by the dye, it might be expected that the AVD of dextran would be smaller than that measured by T 1824, as the larger molecules would have less tendency to traverse the blood vessel. Actually, the AVD of dextran NRC 6 and 7 was less than that of the dye T 1824 by a difference of borderline significance ($p < .05$). The largest dextran fraction did not differ in distribution from the T 1824 space by a significant figure, but the mean difference of 302 ml. smaller volume by dextran NRC 8 is a change of the same order of magnitude observed with dextran NRC 6 and 7. A factor to be considered is that as molecular size is increased possible sequestration of the molecule in phagocytes would increase the measured AVD. Whether or not the slight differences in volume are found to be significant, it is clear that large molecular weight dextrans may be used to measure a volume of distribution similar to that measured by the dye T 1824.

Measurement of dextran in whole blood made possible the estimation of the volume of whole blood in which the amount of injected dextran was dispersed. Although this method obviated the usual estimation of AVD T 1824 in plasma with hematocrit correction for whole blood volume, the results of the 2 methods were not significantly different. If the usual corrections for trapped plasma and body hematocrit were made for the calculated whole blood volume, the result would be somewhat lower than the AVD of dextran in whole blood. Since there is little difference in AVD of dextran or T 1824 in plasma, or possibly a smaller AVD of dextran in plasma, this would suggest that dextran might be measuring a larger red cell volume than was approximated from the theoretical estimation of whole blood volume from AVD of the dye T 1824 in plasma and the observed venous hematocrit.

The observed venous hematocrit was compared with a chemical hematocrit determined by the ratio of concentration of dextran in whole blood and plasma. No significant differences were noted in the results of these 2 hematocrit determinations.

The use of macromolecules of defined molecular size has been shown to present new possibilities for the estimation of intravascular volumes. Several advantages may be realized by the use of this technique since lipemia, hemolysis, serum pigments, staining of tissues, or dangers of radioactivity offer no obstacles in the clinical application. Furthermore, the preparation of substances of defined ranges of molecular size easily measured in blood or body fluids provides a method for the eventual measurement of capillary permeability. Further studies would seem to be indicated for evaluation of this method in experimental and clinical problems.

REFERENCES

1. Rawson, R.: Binding of T 1824 and Structurally Related Azo Dyes by the Plasma Proteins, Am. J. Physiol. **138:** 708, 1943.
2. Schultz, A. L., Hammerstein, A. F., Heller, B. I., and Ebert, R. V.: A Critical Comparison of the T 1824 Dye and Iodinated Albumin Methods for Plasma Volume Measurement, J. Clin. Invest. **32:** 107, 1953.
3. Craig, A. B., Jr., and Waterhouse, C.: The Volume of Distribution of High Molecular Weight Dextran and Its Relation to Plasma Volume in Man, J. LAB. & CLIN. MED. **49:** 165, 1957.
4. Jaenike, J. R., Schreiner, B. F., Jr., and Waterhouse, C.: The Relative Volumes of Distribution of I^{131}-Tagged Albumin and High Molecular Weight Dextran in Normal Subjects and Patients With Heart Disease, J. LAB. & CLIN. MED. **49:** 172, 1957.
5. Gregerson, M. I., and Rawson, R. A.: The Disappearance of T 1824 and Structurally Related Dyes From the Blood Stream, Am. J. Physiol. **138:** 698, 1943.
6. Bloom, W. L., and Willcox, M. L.: Determination of Dextran in Blood and Urine, Proc. Soc. Exper. Biol. & Med. **76:** 3, 1951.

Clubbing of the digits: Physiologic considerations

F. A. BASHOUR, M.D., PH.D. *Dallas, Texas*

Clubbing of the digits occurs in a variety of clinically unrelated conditions. Its mechanism is unknown. In 22 patients with clubbing, 2 physiologic findings were observed: (1) a small peripheral (across the hands) arteriovenous oxygen and carbon dioxide difference indicative of the presence of blood shunting across the hands, and (2) pulmonary veno-arterial admixture. This shunting of blood in both lungs and periphery is believed to occur in the existing arteriovenous anastomoses. It is postulated that a peripheral vasodilator, normally destroyed in the lungs, passes through these shunts unaltered and opens these anastomoses in the digits. Clubbing is believed to result from a reduced oxygen tension in the tissue. In liver cirrhosis, peripheral shunting was observed only in the presence of clubbing or palmar erythema.

Although Hippocratic fingers are frequently encountered in clinical medicine, often accompanied by some other pathologic process,[1] the actual cause of clubbing is obscure. The characteristic deformity of the terminal phalanges is due to an increase in the number and caliber of their blood vessels, together with hypertrophy and hyperplasia of the surrounding fibrous tissue bed.[2] Mendlowitz[3] and Wilson[4] described an increase in blood flow to the digits as a common physiologic factor in such patients. This finding was ascribed to a rise in the digital artery pressure with a decrease in the pressure gradient between the brachial and digital arteries.

In a previous report[5] a case was presented in which a hydatid cyst at the periphery of the lung was associated with desaturated systemic arterial blood and a small arteriovenous oxygen difference across the hand. These findings disappeared following removal of the cyst. Since that time, a number of observations have been made in patients with clubbing of the digits in an attempt to determine the existence of arteriovenous shunting across the hand and veno-arterial shunting through the lungs.

From the Cardiopulmonary Laboratory of the Department of Internal Medicine, The University of Texas Southwestern, Medical School, Dallas, Texas.

Supported by a grant from the American Heart Association.

Presented in part at the Thirty-Second Annual Meeting of the Central Society for Clinical Research, Nov. 7, 1959.

Received for publication Jan. 25, 1961.

The present report is a summary of extended studies designed to elucidate the physiologic nature of circulatory alterations in such patients.

Materials and methods

Twenty-two patients, 7 women and 15 men, with moderate to marked clubbing of the digits were studied. Their ages ranged between 15 and 71 years. The duration of the clubbed fingers could be assessed accurately in 11 patients. None had evidence of pulmonary osteoarthropathy as judged either clinically or by x-ray survey of the bones. The clinical conditions associated with clubbing of the digits in the series are listed in Table I. Case No. 7 was studied before and after steroid therapy with return of the pulmonary function to normal. In Case No. 21, idiopathic clubbing of the digits had been present for 9 years with no evidence of pulmonary disease. In Case No. 22, clubbing of the digits, presumably congenital, had been present all the patient's life. His father and sister also had clubbing of the digits.

In 2 patients, a history of excessive alcohol intake was obtained: In Case No. 6, liver biopsy demonstrated minimal fibroblastic proliferation and periportal cellular infiltration; in Case No. 11, the liver biopsy was normal. In the 2 patients with sarcoidosis, liver biopsy demonstrated nonspecific portal inflammation in one (Case No. 8) and granulomatous infiltration in the other (Case No. 9). In Case No. 5 at postmortem the liver was histologically normal. In the remaining 17 cases there was no clinical evidence of liver disease.

The physiologic studies were carried out at room temperature (24° to 25° C.). All patients were afebrile at the time of study. Blood samples were withdrawn simultaneously from the brachial artery and a forearm vein (labeled in this paper as the cephalic vein). Oxygen and carbon dioxide contents were determined by the method of Van Slyke and Neill. Oxygen tension was determined polarographically at 37° C. from whole blood, using the Clark electrode.[6] From 9 patients, expired air was collected in a Douglas bag, and its oxygen and carbon dioxide concentrations were determined on the Beckman E-2 and Liston-Beckman infrared analyzers, respectively.

Following the initial base-line study with the patient at rest in the supine position and breathing room air, one hand was dipped in ice water (4° to 8° C.) for 1 to 1½ minutes. At the end of this period, arterial and venous blood samples were rapidly withdrawn and analyzed.

Pulmonary venoarterial admixture was determined from the alveolar-arterial oxygen tension gradient after the patient had breathed 100 per cent oxygen for 25 to 30 minutes.[7] The mean alveolar oxygen tension was calculated from the alveolar equation. With pure oxygen breathing and after complete nitrogen washout, the alveolar equation became as follows:

$$PAO_2 = B - P_{H_2O} - PaCO_2,$$

where B = barometric pressure, P_{H_2O} = water vapor tension at 37° C. = 47 mm. Hg, $PaCO_2$ = arterial carbon dioxide tension, assumed to be the same as alveolar CO_2 tension. The arterial CO_2 tension was estimated from the CO_2 content and pH, using the Singer-Hastings nomogram.

Table I. The clinical conditions studied with clubbed fingers

Case No.	*Condition*
1	Hydatid cyst of the left lung
2, 3	Ventricular septal defect (Eisenmenger's)
4, 5	Bronchogenic carcinoma
6	Pulmonary actinomycosis, Ca (?) of the lung
7	Pulmonary eosinophilia
8, 9	Pulmonary and systemic sarcoidosis
10-13	Pulmonary fibrosis and emphysema
14-19	Chronic suppurative lung disease
20	Kyphoscoliosis
21, 22	Idiopathic, congenital

The alveolar-arterial (A-a) oxygen tension gradient is the difference between the mean alveolar oxygen tension (PAO_2) and the arterial oxygen tension (PaO_2), which was determined polarographically.

In calculating the fraction of the blood that is shunted through the lungs, resort is made to an equation the derivation of which has not been published.[9] It is therefore set out in full. The method is based on the fact that the amount of oxygen present in the arterial blood is equal to the amount of oxygen in the blood that has passed through the pulmonary capillaries plus that amount which has by-passed the lungs. This relationship can be expressed by the following formula[8]:

$$CaO_2\dot{Q} = C\acute{e}O_2\dot{Q}_c + C\bar{v}O_2\dot{Q}_s, \qquad 1$$

where CaO_2, $C\acute{e}O_2$, and $C\bar{v}O_2$ represent the oxygen content of arterial blood, end-pulmonary capillary blood, and mixed venous blood, respectively. $\dot{Q}$, $\dot{Q}_c$, and $\dot{Q}_s$ refer to total flow, pulmonary capillary flow, and volume of shunted blood, respectively.

Rearranging symbols for flow and replacing $\dot{Q}_c$ by $(\dot{Q} - \dot{Q}_s)$ in equation (*1*), we obtain:

$$CaO_2\dot{Q} = C\acute{e}O_2\ (\dot{Q} - \dot{Q}_s) + C\bar{v}O_2\dot{Q}_s. \qquad 2$$

Then rearranging:

$$\frac{\dot{Q}_s}{\dot{Q}} = \frac{C\acute{e}O_2 - CaO_2}{C\acute{e}O_2 - C\bar{v}O_2}. \qquad 3$$

Now, adding and subtracting CaO_2 to the denominator:

$$\frac{\dot{Q}_s}{\dot{Q}} = \frac{C\acute{e}O_2 - CaO_2}{C\acute{e}O_2 - CaO_2 + CaO_2 - C\bar{v}O_2}. \qquad 4$$

Further development of the equation requires the assumption that when the subject breathes pure oxygen, end-pulmonary capillary oxygen *tension* ($P\acute{e}O_2$) equals alveolar oxygen *tension* (PAO_2). It is also assumed that end-pulmonary capillary and arterial hemoglobin are fully saturated.

Oxygen *content* of oxygenated samples is obtained by adding the value for oxygen capacity to that of physically dissolved oxygen present in the plasma. This latter value is calculated by multiplying the appropriate oxygen tension by ($f = 0.003$), the solubility factor for oxygen at 37° C. expressed as milliliters per millimeters Hg. Thus, oxygen *content* of end-pulmonary capillary blood is arterial oxygen capacity plus $(PAO_2)f$; oxygen content of arterial blood is arterial oxygen capacity plus $(PaO_2)f$.

Therefore, contents, in the numerator of equation (*4*), can be replaced by tensions, canceling arterial oxygen capacity. In the denominator, the last 2 terms (arterial oxygen content minus mixed venous oxygen content) are the AVO_2 difference.
Hence,

$$\frac{\dot{Q}_s}{\dot{Q}} = \frac{(PAO_2 - PaO_2)f}{(PAO_2 - PaO_2)f + AVO_2\ \text{difference}}. \qquad 5$$

In order to simplify the equation, let *G* equal the alveolar-arterial oxygen tension gradient (in millimeters Hg) multiplied by f: $G = (PAO_2 - PaO_2)f$.
Thus,

$$\frac{\dot{Q}_s}{\dot{Q}} = \frac{G}{G + AVO_2\ \text{difference}}. \qquad 6$$

Ordinarily, this equation will suffice. However, in order to arrive at the particular form previously published,[9] it is necessary to carry this derivation one step further by adding and subtracting (AVO_2 difference) to the numerator, so that:

$$\frac{\dot{Q}_s}{\dot{Q}} = \frac{G + AVO_2 \text{ difference} - AVO_2 \text{ difference}}{G + AVO_2 \text{ difference}} = 1 - \frac{AVO_2 \text{ difference}}{G + AVO_2 \text{ difference}} \quad (7)$$

Equation (7), the final equation, states that the fraction of blood shunted right to left is obtained by subtracting the ratio AVO_2 difference per ($G + AVO_2$ difference) from unity. The per cent right to left shunt is therefore:

$$\text{Per cent R-L shunt} = 100 \left(1 - \frac{AVO_2 \text{ difference}}{G + AVO_2 \text{ difference}}\right).$$

In actual practice, systemic AVO_2 difference (brachial artery and pulmonary artery) is taken to be 4.3 volumes per 100 ml., since the extremes of variance in the arteriovenous oxygen content difference do not alter seriously the estimated degree of shunting.

Results

A. Room air breathing. Table II shows the mean and range of the arterial and arteriovenous difference (across the hands) of oxygen and carbon dioxide in the patients with clubbed fingers and in normal individuals. In the patient with pulmonary eosinophilia (Case No. 7), the study was repeated after restoration of the pulmonary ventilation to normal with steroid therapy; the findings were similar to those of the initial study. A comparatively small arteriovenous oxygen saturation and tension difference was present in patients with clubbed digits as compared to the group without clubbing. There was no overlap between these 2 groups. In 7 patients, the arteriovenous oxygen saturation difference was greater than 10 per cent. Similarly, a small arteriovenous carbon dioxide difference was observed in patients with clubbed digits. In 6 patients, the CO_2 content of the venous blood was lower than that of the arterial blood, as determined by our technique, and in Case No. 18, the CO_2 contents of the venous and the arterial blood samples were equal. Obviously, such a state does not exist in actuality, but since arterial and venous bloods were sampled simultaneously, it is possible that temporal variations occurred with respect to respiration. There was no correlation between the duration or the degree of the clubbing of the digits and the decrease in the arteriovenous difference in oxygen per cent saturation, but the smallest arteriovenous difference appeared in those patients with the shortest history of clubbing.

Table II. Blood gas measurements under ambient conditions*

Items	*Normal*†	*Clubbed fingers*	
		No.	*Mean*
Arterial O_2 saturation (%)	94.9 ± 2.6	23	85.7 ± 9.5
Arterial O_2 tension (mm. Hg)	85 ± 13	18	59 ± 15
Arteriovenous O_2 saturation difference (%)	41.7 ± 12.8	23	7.5 ± 4.6
Arteriovenous O_2 tension difference (mm. Hg)	49 ± 7	18	8 ± 5.5
Arterial CO_2 content (M M/L)	20.4 ± 1.4	17	21.9 ± 4.1
Arteriovenous CO_2 content difference (volume %)	2.03 ± 1.59	17	0.46 ± 0.78
Alveolar-arterial O_2 tension gradient (mm. Hg)	13 ± 6	9	44 ± 18

*At room temperature (24 to 25° C.) ambient oxygen averages 20.85 per cent.

†Normal data reported from this laboratory.[10] (Arteriovenous differences are measured across the hand [brachial artery to cephalic vein]).

Table III shows the peripheral gaseous exchange of oxygen and carbon dioxide in 8 patients with liver cirrhosis. In Cases Nos. 7 and 8 only a small arteriovenous O_2 and CO_2 difference was observed. The patient in Case No. 7 had cirrhosis secondary to chemical toxins with marked palmar erythema and numerous spider nevi extending from the thigh caphalad. In Case No. 8, juvenile cirrhosis and marked clubbing of the digits were present.

Table IV shows the increase in arteriovenous oxygen and carbon dioxide difference upon exposure of the hands to ice water at 4° to 8° C., as compared to that at room temperature (25° C.). In 3 instances (Cases Nos. 8, 11, 18), there was no significant change in the peripheral gaseous exchange upon exposure to cold. In Case No. 8, the O_2 saturation of the venous blood was repeated,

Table III. Peripheral gaseous exchange in patients with cirrhosis of the liver

Case No.	*Arterial oxygen saturation (%)*	*Arteriovenous difference**		
		O_2 (%)	*PO_2 (mm. Hg)*	*CO_2 (vol. %)*
1	90	22	20	+4.18
2	94	29	44	+6.18
3	03	39	44	+3.14
4	81	21	14	+2.78
5	92	30	48	+3.86
6	94	47	37	+2.92
7†	90	7	16	+1.03
8‡	89	4	8	−1.42
Mean	90.37	24.87	30	+2.83

*Arteriovenous differences are measured across the hand (brachial artery to cephalic vein).
†Marked palmar erythema.
‡Clubbed fingers.

Table IV. Peripheral gaseous exchange across the hand upon exposure to cold

Case No.	*Arteriovenous difference**			
	At 25° C.		*At 4 to 8° C.*	
	O_2 (%)	*CO_2 (vol. %)*	*O_2 (%)*	*CO_2 (vol. %)*
5	3	—	19	—
6	1	−0.19	10	+0.09
7A	5	2.18	21	+0.51
7B	5	1.92	14	+5.24
8	3	−1.39	2	−1.97
9	16	1.72	33	+2.47
10	5	0.43	19	+4.66
11	11	0.05	11	0.77
12	6	1.08	20	2.17
13	3	+0.58	20	2.50
18	5	0	4	0.37
19	15	−0.79	22	3.05
21	8	−0.23	13	+1.66
22	13	0.70	26	2.40
Mean	7.0	0.47	16.7	1.84

*Arteriovenous differences are measured across the hand (brachial artery to cephalic vein).

Table V. Pulmonary venoarterial admixture, using the 100 per cent oxygen breathing method

Case No.	*PAO_2 (mm. Hg)*	*PaO_2 (mm. Hg)*	*A-a gradient (mm. Hg)*	*% Shunt*
5	660	239	421	23
6	659	190	489	26
7A	660	344	316	18
7B	666	411	255	15
8	658	384	274	16
9	653	494	159	10
10	655	448	217	13
11	644	361	283	17
13	668	71	597	29
15	608	205	403	22
17	624	131	493	27
18	648	254	394	22
20	657	410	247	15
21A	652	177	475	25
21B	655	185	470	25
22	646	330	316	18
Mean		290	369	20

and proved to be 90 per cent before and 91 per cent after exposure to ice water for 1 to 1½ minutes.

B. Pure oxygen breathing. Table V shows a large alveolar-arterial (A-a) O_2 tension gradient on pure O_2 breathing for 25 to 30 minutes. The arterial O_2 tension (PaO_2) ranged between 71 and 494 mm. Hg. In 2 patients, the A-a oxygen tension gradient was repeated on 2 separate occasions. In Case No. 7, a small decrease from 316 to 255 mm. Hg occurred; in the patient with idiopathic clubbing (Case No. 21), the A-a gradient was the same on both occasions (475 and 470 mm. Hg). In normal subjects, the A-a gradient on 100 per cent O_2 breathing ranged between 0 and 88 mm. Hg in the supine position.[11] The magnitude of the pulmonary venoarterial admixture ranged between 10.0 and 29 per cent of the cardiac output with a mean of 20 per cent. In Case No. 13, as the arterial blood was not completely saturated (PaO_2 71 mm. Hg), the magnitude of the pulmonary venoarterial admixture would be larger than the estimated value. The upper limit of our normal range is 5 per cent.[11]

Discussion

Small arteriovenous oxygen and carbon dioxide differences across the hands were observed in patients with acquired, idiopathic, and congenital clubbed fingers. Harter[12] observed elongated and wide capillaries in these digits in the absence of capillary pulsations. Hall[13] later demonstrated a relatively decreased capillary blood flow, as the rate of absorption of radiosodium from the clubbed fingers was much slower than that from the normal digits. These findings, namely of a small arteriovenous gas difference across the hand, restricted capillary blood flow, and increased blood flow in the hands,[3, 4] suggested the presence

of arteriovenous shunts, presumably through the arteriovenous anastomosis of Sucquet and Hoyer.[14]

These arteriovenous communications occur in large number between the dermis and subcutaneous tissue. They are multilayered, coiled, vascular structures, the lumens of which vary between 20 and 70 μ. They decrease in number from the nail bed to the palmar surfaces of the terminal phalanges to the thenar and hypothenar eminences. They behave like arterioles.[15-17] It is believed that these structures are abundant and appear dilated in the clubbed digits. Dilatation and tortuosity of their collecting venules also have been observed.[4] These vascular structures open and close under the impact of appropriate stimuli. The presence or absence of arteriovenous shunting across the hands depends, therefore, upon the functioning of these arteriovenous anastomoses.

Exposure of the hands to cold water (4° to 8° C.) resulted in an increase in the arteriovenous oxygen and carbon dioxide differences across the hands in patients with clubbed digits, and therefore a decrease of the peripheral shunting. This effect of cold probably was due to constriction of the arteriovenous communications and narrowing of their lumens. In Cases Nos. 8, 11, and 18, the effect of cold was negligible. In Case No. 8, the studies were repeated at another time and again there was no change in the arteriovenous gas difference or in the magnitude of the peripheral shunting. It is reasonable to assume either that in the presence of maximally functioning arteriovenous anastomosis, a slight decrease in the size of their lumens, following exposure to cold, fails to decrease the amount of the blood passing through them, or that in some patients with clubbing of long duration, these structures are no longer responsive to cold stimulus.

The association of clubbing of the digits and liver cirrhosis was described by Fluckiger in 1884 and by Gilbert and Fournier in 1895.[18] Silverstein[19] observed a small arteriovenous oxygen difference in his patients with liver cirrhosis, but Fisher and his co-workers[20] correlated this finding with the presence of palmar erythema in their patients. In our patients with clubbing of the digits, abnormalities in liver function were not found and in only one patient (Case No. 6) was there evidence of early liver cirrhosis.

The study of the peripheral gaseous exchange in our patients with primary liver cirrhosis, on the other hand, demonstrated a small gaseous difference only in the presence of associated clubbing or palmar erythema. This latter finding is believed to precede clubbing in some patients with cirrhosis of the liver,[21] and probably shares a common pathogenesis.

In an earlier observation,[5] pure oxygen breathing failed to saturate systemic arterial blood completely, suggesting the presence of an intrapulmonary right to left shunt. The elevated (A-a) oxygen tension gradient observed in our patients on room air breathing (Table II) may represent the effect of one or more of the following factors: impaired diffusion across the alveolar membrane, hypoventilation of the alveolar spaces, uneven distribution of the inspired air to the alveolar spaces, and pulmonary venoarterial shunting. Breathing pure oxygen for 30 minutes eliminates the alveolar nitrogen, even from the poorly ventilated

areas of the lungs, and brings about an equilibrium between the oxygen tension of the arterial blood and alveolar spaces even if a diffusion defect is present. In the absence of intracardiac right to left shunting, persistence of a large A-a oxygen tension gradient is synonymous with an intrapulmonary venoarterial shunt.

Data in Table V indicate that in all patients with clubbed digits a large A-a oxygen tension gradient is present on 100 per cent oxygen breathing. The degree of intrapulmonary shunting varied from patient to patient, and averaged 20 per cent of the cardiac output. In the 2 patients with idiopathic and congenital clubbing, a large shunt was demonstrated, amounting to 25 and 18 per cent of their respective cardiac outputs. These 2 patients exhibited no demonstrable abnormalities of the lungs.

The intrapulmonary shunting in patients with clubbed fingers occurs presumably through the existing but nonfunctioning arteriovenous communications within the lung.[22-24] These arteriovenous anastomoses are located in the subpleural spaces and around the bronchi. They vary in size from 100 to 390 μ and function when the lung periphery is irritated, such as after the wedging of a cardiac catheter.[25] In patients with primary parenchymatous disease, it is quite conceivable that infection or some irritative mechanism is responsible for opening these arteriovenous anastomoses in the lung. However, in the absence of pulmonary or pleural disease, the mechanism responsible for the opening of the arteriovenous communications in the lungs is obscure.

In 2 reported instances, following the resection of a lung tumor[26] and the removal of hydatid cyst of the lung,[5] the disturbed circulation in both lung and periphery returned to or near normal. One may postulate, therefore, the existence of a reversible mechanism capable of activating arteriovenous shunts in the digits and associated in some way with similar shunts in the lungs.[27]

These observations are in agreement with Beclère's[28] hypothesis, which suggested the possibility of a "chemical substance," normally destroyed in the lungs, that passed through these pulmonary shunts unaltered, producing peripheral vasodilatation. Reduced ferritin may be such a substance.[13] Based on this hypothesis, it is reasonable to assume that reduced tissue oxygen tension secondary to blood shunting, rather than an increased oxygen supply over tissue demand, is the cause of clubbing of the digits.

REFERENCES

1. Mendlowitz, M.: The Digital Circulation, New York, 1954, Grune & Stratton, Inc.
2. Lovell, R.: Observations on the Nature of Clubbed Fingers, Clin. Sc. **9**: 299. 1950.
3. Mendlowitz, M.: Some Observations on Clubbed Fingers, Clin. Sc. **3**: 387, 1938.
4. Wilson, G.: Local Circulatory Changes Associated With Clubbing of the Fingers and Toes, Quart. J. Med. **21**: 201, 1952.
5. Bashour, F.: Hydatid Cyst of the Lung and Clubbing, Ann. Int. Med. (in press).
6. Sproule, B., Miller, W., Cushing, I., and Chapman, C.: An Improved Polarographic Method for Measuring Oxygen Tension in Whole Blood, J. Appl. Physiol. **11**: 365, 1957.
7. Berggren, S.: The Oxygen Deficit of Arterial Blood Caused by Non-Ventilating Parts of the Lung, Acta physiol. scandinav. **4**: 9, 1942.

8. Comroe, J., Forster, R., DuBois, A., Briscoe, W., and Carlsen, E.: The Lung—Clinical Physiology and Pulmonary Function Tests, Chicago, 1955, The Year Book Publishers, Inc.
9. Wilson, R., Ebert, R., Borden, C., Pearson, R., Johnson, R., Falk, A., and Dempsey, M.: The Determinations of Blood Flow Through Non-Ventilated Portions of the Normal and Diseased Lung Am. Rev. Tuberc. **68**: 177, 1953.
10. Sproule, B., Mitchell, J., and Miller, W.: Cardiopulmonary Physiological Responses to Heavy Exercise in Patients With Anemia, J. Clin. Invest. **39**: 378, 1960.
11. Lillehei, J., Johnson, R., Wu, N., Halden, R., and Sproule, B.: Venous-Arterial Admixture in the Lungs in Primary and Secondary Polycythemia, Circulation **17**: 1029, 1958.
12. Harter, J.: Discussion of van Hazel's Article on Joint Manifestations, J. Thoracic Surg. **9**: 505, 1939-40.
13. Hall, G.: The Cause of Digital Clubbing. Testing a New Hypothesis, Lancet **1**: 750, 1959.
14. Masson, P.: Histophysiologie. IV. Les glomus neuro-vasculaires, Paris, 1937, Hermann et Cie.
15. Grant, R., and Bland, E.: Observations on Arteriovenous Anastomosis in Human Skin and in the Bird's Foot With Special Reference to the Reaction to Cold, Heart **15**: 386, 1929-31.
16. Clark, E.: Arteriovenous Anastomosis, Physiol. Rev. **18**: 229, 1938.
17. Hale, A., and Burch, G.: The Arteriovenous Anastomoses and Blood Vessels of the Human Finger. Morphological and Functional Aspects, Medicine **39**: 191, 1960.
18. Gilbert, A., and Fournier, L.: La cirrhose hypertrophique avec ictere chez les enfants, Compt. rend. Soc. biol. **2**: 419, 1895.
19. Silverstein, E.: Peripheral Venous Oxygen Saturation in Patients With and Without Liver Disease, J. Lab. & Clin. Med. **47**: 513, 1956.
20. Fisher, C. J., Eich, R., and Faloon, W.: Arteriovenous Shunting in Palmar Erythema; The Effect Upon Blood Ammonia Determinations, J. Lab. & Clin. Med. **51**: 118, 1958.
21. Bean, W.: Vascular Spiders and Related Lesions of the Skin, Springfield, 1958, Charles C Thomas, Publisher, pp. 88, 288.
22. Prinzmetal, M., Ornitz, E., Simkin, B., and Bergman, H.: Arteriovenous Anastomoses in Liver, Spleen and Lungs, Am. J. Physiol. **152**: 48, 1948.
23. Tobin, C.: The Bronchial Arteries and Their Connections With Other Vessels in the Human Lung, Surg. Gynec. & Obst. **95**: 741, 1952.
24. Sirzi, M., and Bucher, K.: Studies on Arteriovenous Anastomoses in the Lungs, Experimentia **9**: 217, 1953.
25. Rahn, H., Stroud, R., and Tobin, C.: Visualization of Arteriovenous Shunts by Cinefluorography in the Lungs of Normal Dogs, Proc. Soc. Exper. Biol. & Med. **80**: 239, 1952.
26. Semple, T., and McCluskie, R.: Generalized Hypertrophic Osteoarthropathy in Association With Bronchial Carcinoma. A Review Based on 24 Cases, Brit. Med. J. **1**: 754, 1955.
27. Bashour, F. A.: Pulmonary and Peripheral Arteriovenous Shunts in Patients With Clubbed Fingers, J. Lab. & Clin. Med. **54**: 787, 1959. (Abst.)
28. Beclère, M.: Deformation hippocratique des doigts d'une seule main, Bull. et mém. Soc. méd. hôp. Paris **18**: 283, 1901.

Transport of vitamin A across human intestine in vitro

M. R. LORAN, PH.D., T. L. ALTHAUSEN, M.D.,
F. W. SPICER, M.D., and W. I. GOLDSTEIN, B.S.
San Francisco, Calif.

The transport of vitamin A across human small intestine was studied in vitro by means of a modification of the method of Darlington and Quastel. Six specimens of the terminal ileum were obtained directly from the operating table from patients who were undergoing surgery for carcinoma of the right side of the colon. The results were that after 2 hours under aerobic conditions, 6.8 μg per 100 ml. was transferred from the mucosal to the serosal side. No transport occurred under anaerobic conditions or in the presence of 1×10^{-5} M 2,4 dinitrophenol. Chromatographic analysis of intestinal homogenates and the mucosal solution at the end of the perfusion demonstrated that vitamin A palmitate was converted to the alcohol in the lumen or on the surface of the epithelial cells and was then re-esterified in the intestinal mucosa. These results are identical to those previously reported in our transport studies in the rat.

We recently completed a study of the in vitro transport of vitamin A palmitate across normal and remaining resected rat intestine.[1] The results showed that in both normal and resected intestine such transport was an active, energy-requiring process. Chromatographic analysis of vitamin A in the lumen and mucosa of the intestine demonstrated that vitamin A palmitate was de-esterified to the alcohol either in the lumen or at the surface of the mucosal cells. It was then re-esterified in the intestinal mucosa to the acetate or to another short-chain fatty acid similar to acetic acid. This study also indicated that, in the normal intestine, transport of vitamin A depends primarily on energy supplied by oxidative phosphorylation, while in the resected intestine, energy for this purpose is also supplied by anaerobic glycolytic phosphorylation. The purpose of the present project was to construct an apparatus which would permit similar

From the Gastrointestinal Research Laboratory and the Departments of Medicine and Surgery, University of California School of Medicine, San Francisco, Calif.

Supported in part by a grant (Physiol. A-910) from the National Institute of Arthritis and Metabolic Diseases, by funds from the Wine Advisory Board, California State Department of Agriculture, and by the Springer Fund, allocated by the Committee on Research, University of California School of Medicine.

Received for publication Sept. 29, 1960.

experiments on human intestine in vitro and to determine whether the transport of vitamin A in human intestine is similar to that in the rat.

Materials and methods

The transport of vitamin A across sections of human intestine removed at operation was studied by a modification of the perfusion technique of Darlington and Quastel.[2] The perfusion apparatus we designed for this purpose is shown in Fig. 1. It contains 2 independent circulatory systems, one running through the lumen of the intestine, which is designated the *mucosal* circulation, and the other running external to the intestine, which is designated the *serosal* circulation. The solution in the mucosal circulation was composed of calcium-free Ringer bicarbonate buffer containing 0.0056 M glucose, 13 per cent polyoxyethylene sorbitan monooleate (Tween 80) and 330,000 μg per 100 ml. of vitamin A palmitate. The solution in the serosal circulation consisted of calcium-free Ringer bicarbonate buffer and 5 per cent Tween 80 as a solubilizing agent. Circulation of the 2 solutions was maintained by bubbling gas under pressure. The composition of the gas for the aerobic experiments was 93 per cent oxygen and 7 per cent carbon dioxide, and for the anaerobic experiments, 93 per cent nitrogen and 7 per cent carbon dioxide. The pH of both solutions was 7.4. The total volume of each of the solutions bathing the intestine was 1.5 L. The entire system was maintained at 38° C. In some experiments 2,4,dinitrophenol (1×10^{-5} M) was added to the mucosal circulation as a metabolic inhibitor of oxidative phosphorylation.

Measurements were made of the rate of transfer of vitamin A from the mucosal to the serosal circulation. The concentration of vitamin A in the perfusing solutions was determined by the method of Bessey and associates[3]; the rate of transport was reported in μg per 100 ml. per 120 minutes. Determinations of vitamin A in intestinal homogenates were made after the intestine had been thoroughly washed; the concentration was reported in terms of μg per gram. The method for extracting the vitamin A from the intestine and perfusing solutions was previously described.[1] Histologic examination of sections of the intestine after the perfusion experiment showed them to be essentially unchanged by this procedure.

A diphase system of paper chromatography previously described by us[1] was used to characterize the nature of the vitamin A found in the lumen and the mucosal cells after termination of perfusion. Because of the presence of interfering substances in the serosal circulation we have not been able to characterize the form of vitamin A present in this circulation.

Sections of lower ileum, approximately 20 cm. in length, were obtained from patients undergoing surgery because of carcinoma of the right side of the colon. For transportation to the laboratory, the sections were immediately placed in thermos jugs containing warm (37° C.) calcium-free Ringer bicarbonate buffer at pH 7.4, which had previously been saturated with 93 per cent oxygen and 7 per cent carbon dioxide. Perfusion was begun approximately one-half hour from the time of removal. Assay of intestinal homogenates for vitamin A showed that no detectable vitamin A or carotene was present in the intestine prior to perfusion.

Results

As shown in Table I, transport of vitamin A occurred across the human ileum under aerobic conditions. Under anaerobic conditions, however, or in the presence of 2,4,dinitrophenol, which uncouples oxidative phosphorylation, no transport of vitamin A into the serosal circulation took place. Comparison of the data with the results of our previous experiments with rat intestine,[1] as shown in Table II, indicates a close similarity between the manner of transport of this substance in human and in rat intestine. In both species transport of vitamin A was completely inhibited under anaerobic conditions and in the

presence of 2,4,dinitrophenol. This concentration of dinitrophenol (1×10^{-5} M) selectively inhibits phosphorylation reactions that are linked to oxygenation,[4, 5] but does not inhibit the uptake of oxygen by tissues or alter the permeability of the mucosal cells.

Chromatographic separation of vitamin A present in the washings from the lumen of the intestine after perfusion produced 2 spots, one having an R_f value of 0.92, corresponding to that of known vitamin A palmitate, the other having an R_f value of 0.4, corresponding to that of the known vitamin A alcohol. No vitamin A acetate was found in the washings. The vitamin A in the intestinal extract migrated with an R_f value of 0.7, which is similar to that of known vitamin A acetate. No other form of vitamin A was discernible on the chromatogram made with this extract. Chromatographic separation of vitamin A in the experiments in which the intestine was gassed with nitrogen or in which 2,4,dinitrophenol was added to the mucosal solution showed that, although

Table I. Aerobic and anaerobic transport of vitamin A across excised isolated human intestine (ileum)

No. of experiments	*Inhibitor*	*Mean rate of appearance in serosal solution (μg/100 ml./ 120 minutes)*	*Mean content of homogenates (μg/Gm.)*
A. Aerobic (93 % O_2 + 7 % CO_2)			
2	none	6.8	38
2	2,4, dinitrophenol (1×10^{-5} M)	0	2.5
B. Anaerobic (93 % N_2 + 7 % CO_2)			
2	none	0	14

Table II. Aerobic and anaerobic transport of vitamin A across normal rat intestine (ileum)*

No. of experiments	*Inhibitor*	*Mean rate of appearance in serosal solution (μg/100 ml./ 75 minutes)*	*Mean content of homogenates (μg/Gm.)*
A. Aerobic (93 % O_2 + 7 % CO_2)			
9	none	5.31	
5	2,4, dinitrophenol (1×10^{-5} M)	0	
10	none		3.9
5	2,4, dinitrophenol		0.8
B. Anaerobic (93 % N_2 + 7 % CO_2)			
10	none	0	
5	none		0.46

*Previously published results.[1]

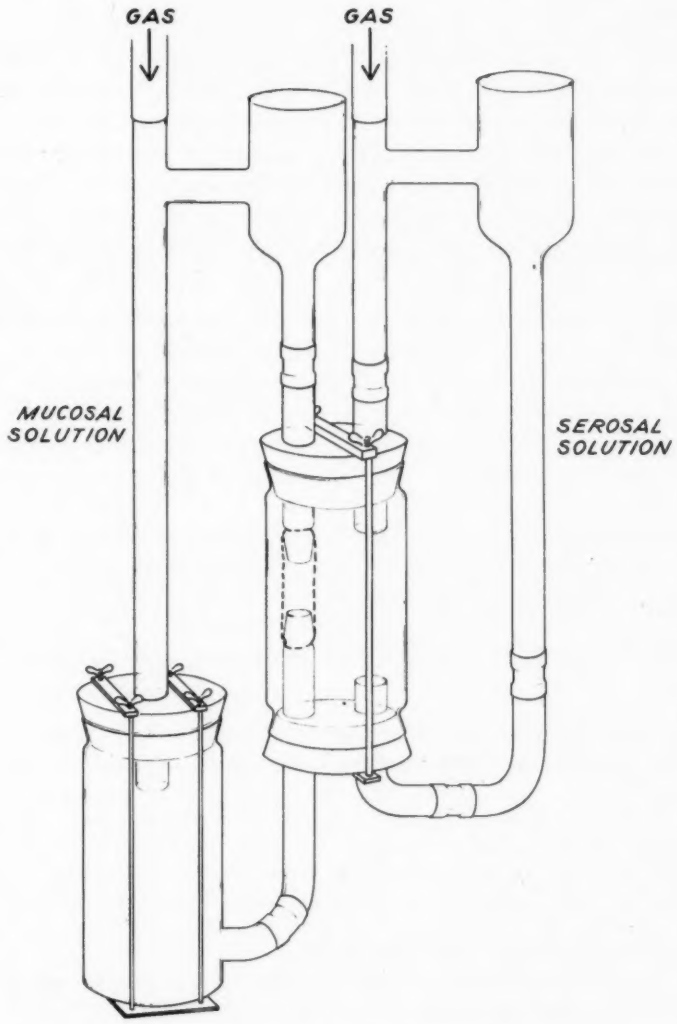


Fig. 1. Diagram of perfusion apparatus used for in vitro studies on human intestine.

vitamin A palmitate was de-esterified to the alcohol in the lumen of the intestine, it was not re-esterified in the mucosa. No vitamin A acetate was found on the chromatogram of the extract of the intestinal homogenate. Therefore, the point at which the inhibitors act is in the acetylation of vitamin A alcohol to vitamin A acetate or to some other short-chain fatty acid ester.

These results indicate that, in man, as in the rat, vitamin A palmitate is de-esterified either on the surface of the mucosal cells or by an enzyme secreted into the mucosal solution; the resultant alcohol is then re-esterified with acetic acid or another short-chain fatty acid as it enters the mucosal cells. The identical results obtained in studies on the ileum of the rat indicate that the mechanism of transport for vitamin A palmitate is the same in the 2 species.

Discussion

A method has been developed for studying the transport of vitamin A across isolated surviving human intestine in vitro. By this method the mechanism of transport of this vitamin was shown to be identical in man and the rat. The outcome of our experiments under aerobic and anaerobic conditions and in the presence of 2,4,dinitrophenol, together with the results of the chromatographic identification of the form of vitamin A present in the lumen of the intestine and in the mucosal cells, supports our previous findings that the transport of vitamin A is an active, energy-requiring process which, in the human and rat intestine, depends primarily on energy supplied by oxidative phosphorylation.

The similarity of the intestinal transport mechanism of vitamin A in the rat and in man indicates that under certain experimental conditions data gained from absorption studies on rat intestine can be applied to man. In the future we hope to study the transport of various nutrients across the human intestine in several pathologic conditions such as celiac disease, sprue, and the post-gastrectomy state.

The described method is applicable not only to studies on the transport of vitamin A but also on the transport of sugars, amino acids, and other substances, under a variety of experimental conditions.

The authors wish to acknowledge the expert technical assistance of Robert Commer.

REFERENCES

1. Loran, M. R., and Althausen, T. L.: Transport of Vitamin A In Vitro Across Normal Isolated Rat Intestine and Intestine Subjected to 'Partial' Resection, Am. J. Physiol. **197**: 1333, 1959.
2. Darlington, W. A., and Quastel, J. H.: Absorption of Sugars From Isolated Surviving Intestine, Arch. Biochem. **43**: 194, 1953.
3. Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A.: Determination of Vitamin A and Carotene in Small Quantities of Blood Serum, J. Biol. Chem. **166**: 177, 1946.
4. Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M., and Gamble, J. L., Jr.: Oxidative Phosphorylation, Science **128**: 450, 1958.
5. Wadkins, C. L., and Lehninger, A. L.: The Adenosine Triphosphate-Adenosine Diphosphate Exchange Reaction of Oxidative Phosphorylation, J. Biol. Chem. **233**: 1589, 1958.

Effect of amino acids on growth and susceptibility to viral hepatitis in mice

BORIS H. RUEBNER, M.D., and KATSUMI MIYAI, M.D.*
Baltimore, Md.

Clinical data suggest the importance of nutrition in liver disease. In human subjects sufficiently rigid dietary experiments rarely can be performed to identify the exact dietary factor involved. The hepatitis produced by M.H.V.$_3$ virus is one of the few available models for human infective hepatitis.

Dietary casein had a protective effect on this infection which was independent of its growth-promoting action.

Neither cystine nor methionine, nor any other essential amino acid alone could reproduce this effect. A mixture of 12 amino acids, however, could substitute for casein in lowering mortality and increasing survival time.

Clinical and epidemiologic observations support the belief that diet is important in the prevention and therapy of liver disease. There is considerable uncertainty, however, about the relative importance of various dietary constituents in particular hepatic diseases.

Since sufficiently rigid dietary experiments usually cannot be performed on man the use of experimental animals becomes desirable. The first prerequisite of such studies is, obviously, an experimental model similar to the human disease. One of the few models for human viral hepatitis is the disease produced by mouse hepatitis virus$_3$ (M.H.V.$_3$) in mice.[1]

Previous work has shown that choline deficiency does not increase the susceptibility of mice to experimental hepatitis. Increasing the dietary protein concentration, however, significantly decreases the mortality from the infection,

From the Department of Pathology, The Johns Hopkins University, Baltimore, Md.
Supported by United States Public Health Service Grant No. E-3598 from the National Institutes of Health, Bethesda, Md.
Received for publication Dec. 20, 1960.
*Fellow supported by United States Public Health Service Training Grant No. 2G-415.

Table I. Composition of experimental diets (percentages by weight)

Dietary constituents	*Diet. No.*											
	1	2	3	4	5	6	7	8	9	10	11	12
Casein	8	12	16	26	40	8	8	8	8	8	8	8
Dextrin	80.5	76.5	72.5	62.5	48.5	80	80	79.5	79	79	79	74.5
Lard	6	6	6	6	6	6	6	6	6	6	6	6
Salt mixture	3	3	3	3	3	3	3	3	3	3	3	3
Vitamin mixture	1	1	1	1	1	1	1	1	1	1	1	1
Cod liver oil	1	1	1	1	1	1	1	1	1	1	1	1
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Alpha tocopherol	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
L cystine						0.46						0.5
DL methionine							0.57	0.57				0.5
DL threonine								0.5				0.5
L arginine									0.5			0.5
L glutamic acid									0.5			0.5
L histidine									0.5			0.5
DL isoleucine										0.5		0.5
DL leucine										0.5		0.5
L lysine										0.5		0.5
DL phenyalanine											0.5	0.5
DL tryptophane											0.5	0.5
DL valine											0.5	0.5

"Vitamin free" casein, white dextrin (technical), Salt Mixture No. 2 U. S. P., and the amino acids were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The vitamin mixture was that of Meader and Williams.[4] Lard was kindly supplied by Esskay Quality Meat Co., Baltimore, Md.

while changes in fat and carbohydrate intake have no effect.[2] In this investigation the effect of dietary protein on mouse hepatitis has been analyzed further. Particular attention has been paid to the relative potency of various concentrations of dietary protein in stimulating growth and in protecting against this infection. The possibility that one individual amino acid in protein might be responsible for this protective effect has also been tested.

Materials and methods

Animals. Female weanling mice of the Swiss Webster strain* were used. Repeated blood examinations have shown these mice to be free from *Eperythrozoon coccoides,* a protozoan which enhances the severity of this infection.[3]

Virus. The M.H.V.$_3$ strain of mouse hepatitis was obtained from Dr. A. W. Gledhill, who originally isolated this virus.[3] It was employed in the form of a suspension in Gey's fluid of mouse liver, recently obtained from infected animals and stored at −30° C. until use. Mortality due to this virus decreases with the age of the animal and depends on the route of inoculation. The concentration of the virus suspension was adjusted so that the intraperitoneal LD_{50} for 32-day-old mice on a 26 per cent protein diet was approximately 0.1 ml.

Diets. The constituents of the experimental diets are shown in Table I.

Procedure. Eighteen-day-old mice were caged in groups of 10 and fed unrestricted quantities of the various diets. Fresh water was supplied daily. The animals were weighed

*Swiss Webster mice obtained from Budd Mountain Farm, Chester, N. J.

individually 3 times weekly. After 14 days they were inoculated intraperitoneally with the same pool of virus suspension. Subsequently, cages were inspected twice daily; dead mice were removed for autopsy.

Results

Effect of changes in casein concentration.

EXPERIMENT 1. Five groups of 30 mice were placed on Diets 1 through 5 (Table I) which varied in casein concentration from 8 to 40 per cent. At the start of the experiment the mean weights of the various groups (7.8 to 8.7 grams) did not differ significantly. After 14 days the increase in the average weights of the different groups ranged from 3.8 to 10.7 grams (Table II). The mice were then injected with 0.1 ml. of a 1:100 suspension of liver from infected animals.

The number of mice in the various groups dying each day after injection is shown in Table II. Mortality was inversely and mean survival time directly related to the dietary casein concentration. There was a straight-line relationship between the log of the dietary casein concentration and the percentage of the survivors expressed on a probit scale (Fig. 1). The difference in mortality between the diets with the lowest and the highest casein concentration (Diets 1 and 5) was highly significant ($X^2 = 11.1$; $p = < 0.01$). The mean survival time was also significantly prolonged in the animals receiving 40 per cent casein as compared with the 8 per cent casein group ($t = 3.4$; $p = < 0.01$). The growth rate, however, was virtually optimal in the animals receiving 16 per cent casein and increase of the dietary casein beyond this level produced no further increase in the weights of the animals. Since the differences in mortality between the 3 diets producing optimal growth (Diets 3, 4, and 5) were small, it was decided to feed them to larger groups of animals in the next experiment.

Table II. Number of mice dying each day after injection

Diet No.	*Mean growth (Gm.)*	*Days after injection*										*Total deaths*	*Mean survival (days)*
		1	2	3	4	5	6	7	8	9	10		
1	3.8	0	0	2	15	7	3	0	0	0	0	27	4.4
2	8.2	0	0	0	9	14	1	2	0	0	0	26	4.8
3	9.8	0	0	0	7	9	4	0	1	0	0	21	5.0
4	10.7	0	0	0	2	13	1	4	0	0	0	20	5.3
5	9.4	0	0	0	2	10	4	1	0	1	0	18	5.4

Table III. Number of mice dying each day after injection

Diet No.	*Mean growth (Gm.)*	*Days after injection*										*Total deaths*	*Mean survival (days)*
		1	2	3	4	5	6	7	8	9	10		
3	9.6	0	0	3	10	14	8	1	0	0	0	36	4.8
4	9.2	0	0	0	7	14	7	2	1	0	0	31	5.2
5	9.5	0	0	1	0	10	4	6	0	0	1	22	6.0

EXPERIMENT 2. Three groups of 50 mice were placed on Diets 3, 4, and 5, which contained 16, 26, and 40 per cent casein, respectively (Table I). After 14 days, when the average growth was almost identical in the 3 groups (Table III), the mice were injected with 0.1 ml. of a 1:30000 liver suspension. This virus preparation, due to passage, was more virulent than that employed in the previous experiment.

The number of mice in the 3 groups dying each day after injection is shown in Table III. Although growth of all groups was similar, mortality was still inversely, and mean survival time directly, related to the casein concentration. The diminution in the number of deaths ($X^2 = 8.9$; $p = < 0.01$) and the increase in the mean survival time ($t = 4.0$; $p = < 0.001$) produced by the 40 per cent casein diet (Diet 5) in comparison with the 16 per cent casein diet (Diet 3) were both highly significant. It was concluded from this experiment that the protective effect of casein in mouse hepatitis is independent of its growth-stimulating action.

Effect of supplementation with amino acids.

EXPERIMENT 3. Five groups of 30 mice were placed on Diets 1, 4, 6, 7, and 8 (Table I). Diet 1 was low in casein (8 per cent). Diet 4, high in casein (26 per cent), was included in this experiment as a control group. Diets 6 and 7 were identical to the low-casein diet with the exception of the addition of cystine in Diet 6 and methionine in Diet 7, in amounts equivalent to the sulfur-containing amino acids in the high-casein diet. The quantities to be added were calculated from the data of Himsworth and Glynn[5] and of Rose and Wixom.[6] Diet 8 consisted of 8 per cent casein supplemented with methionine and threonine. In a casein diet, threonine, apart from the sulfur-containing amino acids, is the next most important amino acid limiting growth.[7] After 14 days the growth of the groups supplemented with amino acids was only slightly greater than that of the animals receiving the unsupplemented low-casein diet (Table IV). Only Diet 8, containing both methionine and threonine, produced significantly greater growth than the low-casein Diet 1 ($t = 2.6$; $p = < 0.05$).

Mortality and mean survival time (Table IV), after injection of a 1:100000 liver suspension, were unaffected by supplementation of the low-casein diet with cystine alone (Diet 6), with methionine (Diet 7), or with both methionine and threonine (Diet 8). Diet 4, the high-casein control diet, again produced a strikingly lowered mortality and increased mean survival time.

Table IV. Number of mice dying each day after injection

Diet No.	*Mean growth (Gm.)*	*Days after injection*										*Total deaths*	*Mean survival (days)*
		1	2	3	4	5	6	7	8	9	10		
1	2.0	0	0	1	7	17	1	0	0	0	0	26	4.7
4	11.3	0	0	0	1	8	3	3	0	0	0	15	5.5
6	2.5	0	0	3	9	10	2	0	0	0	0	24	4.4
7	3.5	0	0	1	14	10	1	0	0	0	0	26	4.4
8	3.8	0	0	3	14	9	0	0	0	0	0	26	4.2

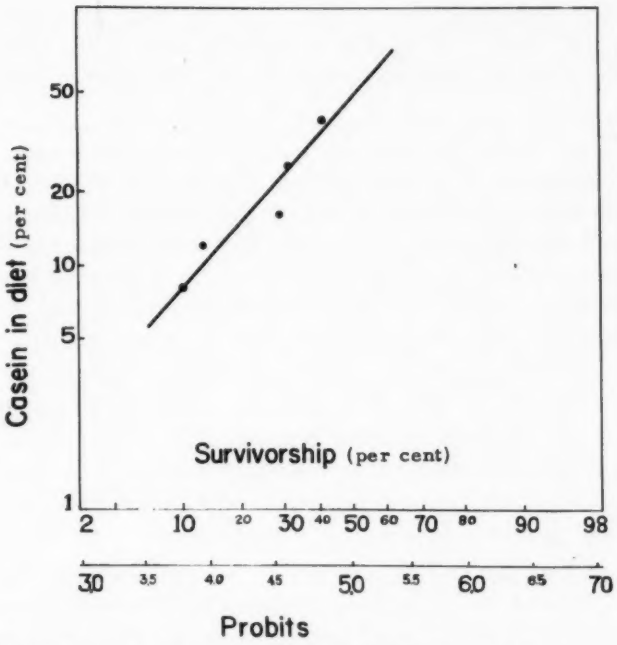


Fig. 1. Effect of dietary casein on mortality from M.H.V.$_3$ hepatitis.

EXPERIMENT 4. Six groups of 30 mice were fed Diets 1, 4, 9, 10, 11, and 12 (Table I). Diets 1 and 4 were the low- and high-casein diets, respectively, employed in the previous experiment. Diets 9, 10, and 11 were identical with the low-casein diet except for the addition of groups of 3 amino acids to each diet. Diet 12, in addition to 8 per cent casein, was supplemented with 0.5 per cent of each of 12 amino acids, including all those usually considered essential.[7] After 14 days the growth of the animals on diets low in casein and supplemented with 3 amino acids (Diets 9, 10, and 11) was no greater than that of the group receiving Diet 1, the unsupplemented low-casein diet (Table V). After injection of a 1:100000 liver suspension, mortality and mean survival time of animals on these supplemented diets also were not significantly changed.

Only Diet 12, which was supplemented with 12 amino acids, produced a significant increase in growth ($t = 5.2$; $p = < 0.001$). This regimen also sig-

Table V. Number of mice dying each day after injection

Diet No.	*Mean growth (Gm.)*	*Days after injection*										*Total deaths*	*Mean survival (days)*
		1	2	3	4	5	6	7	8	9	10		
1	4.2	0	0	3	13	9	3	1	0	0	0	29	4.3
4	9.4	0	0	1	7	8	3	3	0	0	0	22	5.0
9	4.2	0	0	5	15	6	1	0	0	0	0	27	4.1
10	4.1	0	0	0	11	10	2	0	0	1	0	24	4.6
11	3.8	0	0	2	8	13	1	1	0	0	0	25	4.6
12	6.3	0	0	0	1	15	3	1	0	0	0	20	5.2

nificantly reduced mortality ($X^2 = 7.1$; $p = < 0.01$) and prolonged survival in comparison with Diet 1 ($t = 2.7$; $p = 0.01$). Diet 4, the high-casein control diet, produced a mortality and mean survival time similar to that of Diet 12.

Discussion

The chief finding in this investigation was that increasing the dietary protein concentration beyond the optimal level for growth still further reduced the mortality from mouse hepatitis (Table II). There was also a significant, if small, increase in mean survival time. Since mortality was lowered and survival time increased without any effect on growth, it is concluded that protein exerts a true protective effect. This is an unusual finding in experimental virus diseases where dietary deficiencies usually render animals relatively insusceptible.[8] In epidemics of human viral hepatitis, however, most observers have considered that prior malnutrition may increase mortality.[9-12] In the light of our experimental results it seems probable that protein deficiency was responsible for the severity of virus hepatitis in those malnourished animals. Many clinical observers believe also that cautious forcing of a high-protein diet is beneficial therapeutically.[13]

Adding a nutrient to an already adequate diet rarely has been shown to benefit infections.[8] Schneider's salmonellosis resistance factor, however, possesses this property. Plotting the log of the concentration of this factor against the percentage of survivors on a probit scale resulted in a straight-line relationship.[14] We found a similar correlation between the dietary protein concentration and the percentage of survivors after infection with experimental mouse hepatitis (Fig. 1). Further search may well lead to the identification of other nutrients which, if added to a diet already adequate for growth, are still capable of protecting against an infectious disease.

Cystine, methionine, and threonine are the amino acids which limit growth on low-casein diets.[7] In the present study cystine and methionine were added to the low-protein diet in amounts calculated to be equivalent to the concentration of sulfur-containing amino acids of the high-casein diet and threonine was added at an arbitrary level of 0.5 per cent. The effect of these amino acids on growth was less than anticipated (Table III), and, under the conditions of our experiments, other amino acids were apparently almost equally important in limiting growth. Addition of cystine, methionine, and threonine did not decrease the severity of experimental mouse hepatitis. Supplementation of diets with sulfur-containing amino acids has long been considered beneficial in certain types of liver injury.[15] There are, however, nutritional disorders involving the liver, such as kwashiorkor, where a deficiency of sulfur-containing amino acids has not been implicated any more than has lack of other essential amino acids. There also does not seem to be any evidence that the sulfur-containing amino acids are responsible for the benefits produced by dietary protein in certain bacterial infections.[8] In the light of these observations the inability of cystine and methionine to protect mice against experimental viral hepatitis becomes less surprising.

In the final experiment, 9 further amino acids, at an arbitrary concentration of 0.5 per cent, were tested for capacity to reproduce the protective effect of casein. In order not to use excessive numbers of experimental animals these 9 amino acids were alphabetically divided into groups of 3. Three groups of mice were fed low-casein diets, each of which was supplemented with 3 amino acids. These supplements did not produce an increase in mean weight and scarcely affected mortality and survival time (Table V). Obviously, none of these 9 amino acids alone was responsible for the full protective potency of casein. Supplementation of the low-casein diet with all these 9 amino acids as well as with cystine, methionine, and threonine did, however, result in a significantly lower mortality and increased survival. It is concluded that an amino acid mixture can substitute for casein, not only in stimulating growth, but also in protecting against experimental hepatitis.

The authors wish to thank Dr. H. A. Schneider for much helpful advice and M. Dabney and G. Hoffman for their excellent technical assistance.

REFERENCES

1. Ruebner, B. H., and Bramhall, J. L.: The Pathology of Experimental Virus Hepatitis in Mice, Arch. Path. **69**: 190, 1960.
2. Ruebner, B. H., and Bramhall, J. L.: The Effect of Dietary Protein on Experimental Virus Hepatitis, Gastroenterology **39**: 335, 1960.
3. Dick, G. W. A., Niven, J. S. F., and Gledhill, A. W.: A Virus Related to That Causing Hepatitis in Mice (M.H.V.), Brit. J. Exper. Path. **37**: 90, 1956.
4. Meader, R. D., and Williams, W. L.: Choline Deficiency in the Mouse, Am. J. Anat. **100**: 167, 1957.
5. Himsworth, H. P., and Glynn, L. E.: The Prevention of Experimental Massive Necrosis by Methionine, Clin. Sc. **5**: 133, 1944.
6. Rose, W. C., and Wixom, R. L.: The Amino Acid Requirements of Man, J. Biol. Chem. **216**: 763, 1956.
7. Harper, A. E.: Sequence in Which the Amino Acids of Casein Become Limiting for the Growth of the Rat, J. Nutrition **67**: 109, 1959.
8. Scrimshaw, N. S., Taylor, E., and Gordon, E.: Interactions of Nutrition and Infection, Am. J. M. Sc. **237**: 367, 1959.
9. Himsworth, H. P.: Lectures on the Liver and Its Diseases, ed. 2, Harvard University, Cambridge, Mass., 1950, 120.
10. Hahn, R. G., and Bugher, J. C.: Observations During an Outbreak of Infectious Hepatitis in British West Africa, Tr. Roy. Soc. Trop. Med. & Hyg. **48**: 77, 1954.
11. Hoffman, H.: Statistische Untersuchungen über den Normalverlauf der Virus Hepatitis, Dtsch. Z. Verdauungsk. **17**: 70, 1957. Abstracted in Gastroenterology **34**: 164, 1958.
12. Findlay, G. M.: Infective Hepatitis in West Africa. II. Syringe Transmitted Hepatitis, Month. Bull. Min. Health, **7**: 32, 1948.
13. Chalmers, T. C.: An Evaluation of Dietary Protein in the Treatment of Liver Disease, Ann. Int. Med. **48**: 320, 1958.
14. Schneider, H. A.: Nutritional Factors in Host Resistance, Bact. Rev. **24**: 186, 1960.
15. Hartroft, W. S.: Experimental Hepatic Injury in Diseases of the Liver, L. Schiff, editor, Philadelphia and Montreal, 1958, J. B. Lippincott & Co., p. 92.

A comparative study of monkey kidney cell cultures and suckling mice for the recovery of enteroviruses from patients with central nervous system disease

EDWIN H. LENNETTE, M.D., PH.D., ANNA WIENER, B.S., ISAO HOSHIWARA, M.D.,* JAMES WOODIE, M.S., and ROBERT L. MAGOFFIN, M.D. *Berkeley, Calif.*

A comparative study of the use of monolayer cultures of monkey kidney cells and of 1-day-old mice for the primary isolation of enteroviruses was made on a series of 1,128 specimens from 460 patients with suspected viral disease of the central nervous system. A total of 366 specimens from 188 patients yielded a virus; from 57 per cent of the specimens an isolation was made, in both monkey kidney cells and suckling mice, from 29 per cent in the former and 14 per cent in the latter. In only 2 instances were different viruses recovered from the same patient in each system. Thirteen patients yielded Coxsackie A viruses (types 2 through 7) in mice only, and 5 (4 per cent) of 114 patients infected with a Group B Coxsackie virus yielded virus solely in suckling mice. Of the remaining Coxsackie B infections, 101 (89 per cent) were detected in monkey kidney cells and suckling mice, and 8 (7 per cent) in monkey kidney cells only. Altogether, specimens from 20 patients, about 10 per cent of those yielding a virus, or 5 per cent of the entire study group, yielded a virus in suckling mice that was not recovered in monkey kidney cells. Eleven different types of ECHO viruses were recovered in monkey kidney cells from 32 patients, and 3 of 17 strains of ECHO 9 virus also were recovered in suckling mice. Polioviruses were recovered from 16 patients, solely in monkey kidney cells. It is suggested that, for laboratory diagnostic purposes in cases of suspected viral central nervous system disease, 1-day-old mice may be used as an adjunct to monkey kidney cells rather than as a duplicate procedure.

Although monolayer cultures of monkey kidney cells (MK) are suitable for the primary isolation of a broad range of enteroviruses including poliomyelitis, ECHO, Coxsackie Group B viruses, and type 9 of the Group A Coxsackie viruses, the recovery of most types of Group A Coxsackie viruses requires

From the Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley, Calif.

Supported by Grant No. (E-1475) from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

Received for publication Jan. 26, 1961.

*Formerly Medical Officer, Laboratory Branch, Communicable Disease Center, assigned to the Viral and Rickettsial Disease Laboratory.

the use of suckling mice (SM).[1] The Group B Coxsackie viruses and certain strains of several other types of enteroviruses, e.g., Coxsackie A9, A16, and ECHO 9, may be recovered in both MK and SM, but among the latter viruses a decided variation has been noted between strains in their relative pathogenicity for one or the other host systems.[1-3] Etiologic studies conducted in this laboratory over the past several years to assess the association of enterovirus infections with suspected viral disease of the central nervous system[4] afforded an opportunity to compare the viral isolations made by each method from a series of 1,128 stool specimens representing 460 patients. Observations were made with several objectives in mind: (1) to compare the over-all spectrum of virus types recovered by each method; (2) to assess the relative effectiveness of the 2 systems for the primary isolation of specific viral types which are infective for both MK and SM; (3) to look for evidence of concurrent infection with 2 or more viruses, and specifically for instances in which each isolation method would detect different viruses from the same patient; and (4) to assess the merits of the suckling mouse as an adjunct to tissue culture and serologic tests which might be included among the laboratory diagnostic procedures employed in cases of suspected viral central nervous system disease.

Materials and methods

Source of specimens. The majority of specimens were collected from patients with various clinical types of suspected viral central nervous system disease observed in the Communicable Disease Unit of the Los Angeles County General Hospital during the summer months of 1957 and 1958. Two or 3 stools and serial blood specimens were obtained from each patient. Stools were usually held at 4° C. at the hospital and shipped weekly by air express to the laboratory, where they were kept frozen at –10° C. until tested. A few of the specimens (less than 10 per cent) were from patients hospitalized in the Oakland area. Each year stools from approximately 200 patients were tested in parallel in MK and SM without reference to the findings; this number was supplemented by cases, predominantly of paralytic illness, in which isolation attempts in SM were undertaken after stools had failed to yield a virus in MK. Altogether, a series of 1,128 stools from 460 patients was tested in both MK and SM.

Isolation and identification of viral agents. For viral isolation attempts, 10 per cent stool suspensions were inoculated into monkey kidney cell cultures as well as into infant mice less than one day of age. The procedure for viral isolation in monkey kidney cell cultures has been described previously.[5] Two litters (6 each) of infant mice less than 24 hours of age were inoculated intracerebrally with 0.01 ml., as well as intraperitoneally with 0.03 ml., of the processed stool suspension. Mice were examined daily over a 14 day observation period for evidence of infection, and those showing signs of infection were sacrificed and the brains and muscles harvested for passage and identification.

All viral agents isolated in monkey kidney cell cultures were first screened in a colorimetric identification scheme[6] against immune sera to the 3 poliovirus types and the Group B Coxsackie viruses, types 1 through 5. Isolates which could not be identified in this system were tested against Coxsackie A9 and ECHO immune sera in neutralization tests employing monolayer cultures of MK. Viruses recovered in suckling mice were also screened (using infected mouse tissue) in the colorimetric neutralization test for Group B Coxsackie virus types 1 through 5; agents which could not be identified by this procedure were identified by neutralization tests done in SM.

Serologic tests. Complement fixation tests for poliomyelitis, mumps, herpes simplex, St. Louis encephalitis, and Western equine encephalomyelitis viruses were performed by the

basic procedure described elsewhere.[7] Acute- and convalescent-phase sera from 200 patients in this study were also assayed for neutralizing antibodies to Group B Coxsackie virus types 1 through 5 by the colorimetric procedure based upon the use of FL-line human amnion cells.[6]

Results

The clinical diagnoses in the 460 patients on whom laboratory studies were done are presented in Table I together with laboratory evidence of the virus involved. The clinical and etiologic considerations concerning the association of particular viruses with each category of central nervous system disease have been discussed elsewhere[4] and will not be dealt with here. Table I is included here, however, to indicate the clinical composition of the study group and to summate the laboratory findings from which the comparison of SM and MK results was drawn. Cases clinically classified as aseptic meningitis comprised about 70 per cent of the group, with the remainder divided among clinical diagnoses of paralytic poliomyelitis (55 cases), encephalitis (46 cases), and a miscellaneous group of other diagnoses (29 cases), e.g., "Guillain-Barré syndrome," "acute myelitis," "acute febrile illness, and possible central nervous system involvement." It should be noted that the selection of cases of paralytic poliomyelitis in this study was not a representative selection but was weighted by the inclusion of patients with paralytic illness whose stools were tested in suckling mice when poliovirus infection could not be confirmed.

Table I. Clinical diagnosis and laboratory evidence of etiology in 460 patients with suspected viral central nervous system disease

Laboratory evidence of etiology	*No. of patients*	*Clinical diagnosis*			
		Paralytic poliomyelitis	*Aseptic meningitis*	*Enceph-alitis*	*Other diagnoses*
Virus isolated					
Poliovirus	16	15	–	1	–
Coxsackie B2-B5	114	3	106	3	2
Coxsackie A9	4	–	4	–	–
Coxsackie A2-A7	13	–	9	4	–
ECHO	32	1	30	1	–
Unidentified	3	–	3	–	–
Dual isolation	6	2*	4*	–	–
No virus isolated					
Antibody titer rises to:					
Poliovirus	4	–	4	–	–
Coxsackie B1-B5	15	1	12	2	–
Mumps†	23	2	16	5	–
Herpes simplex	7	1	3	1	2
St. Louis encephalitis†	1	–	–	1	–
Undetermined	222	30	139	28	25
Total	460	55	330	46	29

*See Table III.
†Fourfold or greater rise in antibody titer or convalescent-phase titer of 1:64 or more.

Group A Coxsackie viruses were not recovered, either alone or in association with a poliovirus, from patients with paralytic illness, and were only occasionally isolated from cases in other clinical categories. The high incidence of Group B Coxsackie infections encountered in cases of aseptic meningitis provided suitable data for comparing the effectiveness of the 2 isolation methods for the recovery of this group of viruses, but among the array of other enteroviruses isolated

Table II. Comparison of monkey kidney cell cultures (MK) and 1-day-old suckling mice (SM) for recovery of enteroviruses from stools of 460 patients with central nervous system disease

Results of isolation attempts	*No. of patients*	*Stools tested*	*Number of stools yielding virus*			
			Total	*MK and SM*	*MK only*	*SM only*
Virus isolated	*188*	*442*	*366*	*210* (57%)	*105* (29%)	*51* (14%)
Poliovirus	*16*	*33*	*29*	–	*29*	–
1	6	21	17	–	17	–
2	3	4	4	–	4	–
3	7	8	8	–	8	–
Coxsackie B	*114*	*273*	*240*	*199* (83%)	*18* (8%)	*23* (10%)
B2	3	5	5	4	–	1
B3	2	5	4	4	–	–
B4	41	82	79	66 (84%)	4 (5%)	9 (11%)
B5	68	181	152	125 (82%)	14 (9%)	13 (9%)
Coxsackie A9	*4*	*6*	*6*	*2*	*4*	–
Coxsackie A	*13*	*29*	*26*	–	–	*26*
A2	4	7	6	–	–	6
A3	1	3	3	–	–	3
A4	1	2	2	–	–	2
A5	1	3	3	–	–	3
A6	1	3	1	–	–	1
A7	5	11	11	–	–	11
ECHO	*32*	*80*	*49*	*3*	*46*	–
2	1	3	2	–	2	–
4	6	19	10	–	10	–
6	5	11	6	–	6	–
7	1	3	1	–	1	–
9	12	27	17	3 (18%)	14 (82%)	–
10	2	5	3	–	3	–
11	1	2	2	–	2	–
14	1	3	2	–	2	–
17	1	2	2	–	2	–
18	1	3	2	–	2	–
19	1	2	2	–	2	–
Unidentified	*3*	*5*	*4*	–	*2*	*2*
Dual isolation	*6*	*16*	*12*	*6*	*6*	–
No virus isolated	272	686				
Totals	460	1,128	366	210	105	51

there were relatively few representatives of any single immunotype. Serologic tests detected 4 poliovirus infections and 15 infections with a Group B Coxsackie virus which were missed by isolation attempts. An additional 31 patients whose stools did not yield virus had serologic evidence of a nonenteric virus infection, i.e., mumps, herpes simplex, or St. Louis encephalitis.

The results of attempted virus isolation from 1,128 fecal specimens in MK cultures and SM are shown in Table II. A total of 366 specimens, representing 188 patients, yielded a virus in one or both systems. Isolations were made from 210 specimens in both MK and SM, from 105 in MK only, and from 51 in SM only. Six patients yielded 2 different immunotypes of virus, in 3 instances from the same specimen. To simplify presentation of the data, these instances of dual isolations are shown as a single group in Table II, and are described individually in Table III. A total of 318 isolations was made in MK and 261 in SM.

Poliovirus was recovered from 16 patients, solely in MK; of 33 stools tested from these 16 patients, the virus was recovered from 29, or 88 per cent. Group B Coxsackie viruses were recovered from 240 specimens, of which 83 per cent yielded virus in both MK and SM, 8 per cent in MK only, and 10 per cent in SM only. Thus, either isolation method alone was successful in detecting approximately 90 per cent of the strains recovered by the use of both systems. With respect to individual immunotypes of Coxsackie viruses, the results were essentially similar for types B4 and B5. The few strains of types B2 and B3 encountered were recovered in both systems, except one specimen which yielded B2 in SM only. Type B1 was not encountered.

Table III. Comparative recovery of enteroviruses in monkey kidney cell cultures (MK) and suckling mice (SM). Stools are from 6 patients who yielded more than one type of virus

Patient No.	*Clinical diagnosis*	*Stool specimen*					
		First		*Second*		*Third*	
		MK	*SM*	*MK*	*SM*	*MK*	*SM*
1	Aseptic meningitis	Polio 1 and ECHO 4	Negative	ECHO 4	Negative		
2	Aseptic meningitis	Polio 1 and "X"	Negative	Negative	Negative	Negative	Negative
3	Spinal paralytic poliomyelitis	Polio 1	Negative	Polio 1 and Coxsackie B5	Negative	Polio 1	Negative
4	Aseptic meningitis	Negative	Negative	Coxsackie B5	Coxsackie B5	ECHO 10	Coxsackie B5
5	Spinal paralytic poliomyelitis	Coxsackie B4	Coxsackie B4	Coxsackie B5	Coxsackie B5		
6	Aseptic meningitis	"X"	Coxsackie A1	"X"	Coxsackie A1	Negative	Negative

"X" = unidentified.

Of the Group A Coxsackie viruses, type A9 was recovered in MK from 6 specimens, but only 2 of these also yielded the virus in SM. A total of 26 specimens yielded other Group A viruses, types 2 through 7, and in all instances recovery was made in SM only. In addition, 2 successive stools from one patient (Table III) yielded a Coxsackie A1 virus in SM and an unidentified ECHO virus in MK.

ECHO viruses of 11 different types (2, 4, 6, 7, 9, 10, 11, 14, 17, 18, 19) were recovered from 49 specimens. From 3 of 17 specimens yielding ECHO type 9, the virus was recovered in SM as well as MK. In all other instances, ECHO viruses were recovered solely in MK.

Four specimens (3 patients) yielded enteroviruses which have not yet been identified. Two of these unidentified isolates were recovered solely in SM, producing characteristic pathologic changes indicative of Group A Coxsackie viruses. The other 2 unidentified strains were recovered solely in MK and are presumed to be ECHO viruses which could not be typed with available immune sera.

The frequency of "negative" stools interspersed with one or more positive specimens from the same patient may be seen in Table II by comparing the number of stools tested with the number yielding virus. Among patients yielding Group A or B Coxsackie viruses or a poliovirus, virus was recovered from approximately 85 to 90 per cent of the specimens tested, whereas, among patients yielding ECHO viruses, about 40 per cent of the specimens were negative. Since the specimen collection schedule was essentially similar for patients in each isolation category, the less consistent recovery of ECHO viruses from consecutive stools suggests either that there was greater variation in the excretion of these viruses or that they are less readily detected in MK than are the Coxsackie and polio viruses.

Table IV. Comparative recovery of enteroviruses in monkey kidney cell cultures (MK) and suckling mice (SM). Patients are those with suspected viral central nervous system disease

	Patients from whom virus was recovered in							
	Total		*MK and SM*		*MK only*		*SM only*	
Types of viruses recovered	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Total patients tested	460	100	108	23	61	13	19	4
Total yielding virus	188	100	108	57	61	32	19	10
Poliovirus	16	100	–	0	16	100	–	0
Coxsackie B	114	100	101	89	8	7	5	4
Coxsackie A9	4	(100)	1	(25)	3	(75)	–	(0)
Coxsackie A2-A7	13	100	–	0	–	0	13	100
ECHO	32	100	3	9	29	91	–	0
Unidentified	3	(100)	–	(0)	2	(67)	1	(33)
Dual isolation	6	(100)	3	(50)	3	(50)	–	(0)
No virus recovered	272	100	–	–	–	–	–	–

Percentages are rounded independently and may not add to totals; parentheses indicate percentages based on less than 10 cases.

Table V. Effect of examination of multiple stool specimens on proportion of enterovirus infections detected

Type of virus isolated	*Isolation method*	*No. of patients*	*Number of infections detected by testing*			*Accumulative per cent of infections detected*		
			First stool	*Second stool*	*Third stool*	*First stool*	*Second stool*	*Third stool*
Poliovirus	Tissue culture	9	8	0	1	89	89	100
Coxsackie A2-A7	Suckling mice	11	10	1	0	91	100	100
Coxsackie B	Tissue culture	87	80	4	3	92	97	100
Coxsackie B	Suckling mice	86	83	2	1	97	99	100
Coxsackie B	Tissue culture or suckling mice	91	88	1	2	97	98	100
ECHO virus	Tissue culture	27	18	4	5	67	81	100

The finding of more than one type of enterovirus associated with illness was relatively infrequent in this study. It should be pointed out that the presence in the same specimen of an ECHO virus and a Coxsackie B or polio virus could have gone undetected by our procedure of first identifying isolates of the latter 2 groups of viruses colorimetrically in FL-line amnion cells and then secondarily undertaking typing in monolayer MK cultures of those isolates which could not be readily passed in FL cells. These data should provide a valid indication, however, of multiple infections detected by the use of both MK and SM which would have been missed had only one or the other isolation procedure been employed. There were only 2 such instances (Table III): the recovery of ECHO type 10 in tissue culture and Coxsackie B5 in SM from one patient (Case No. 4); and the recovery from another (Case No. 6) of Coxsackie A1 in SM and an unidentified enterovirus in tissue culture.

Table VI. Comparison of monkey kidney cell cultures, suckling mice, and in vitro neutralizing antibody determinations for the laboratory diagnosis of Group B Coxsackie virus infection

Laboratory results	*Total*		*Virus type*				
	No.	*%*	*B1*	*B2*	*B3*	*B4*	*B5*
Total patients	95	100%					
Virus recovered in monkey kidney cells	80	84%	–	3	2	21	54*
Virus recovered in suckling mice	77	81%	–	3	2	21	51
Virus recovered in monkey kidney cells and/or suckling mice	82	86%	–	3	2	21	56
≥ Fourfold rise in homotypic antibody	42	44%	–	1	1	11	29
< Fourfold rise in homotypic antibody titer ≥ 1:32	38	40%	–	2	1	10	25
titer < 1:32	2	2%	–	–	–	–	2
Virus not recovered, ≥ fourfold rise in antibody titer	13†	14%	(2)	(2)	(2)	(2)	(11)

*Coxsackie B4 virus also isolated from one patient.

†Since rises in antibody titer to multiple viral immunotypes occurred in 4 patients, the figures do not add to the total.

A comparison of the infections detected by each isolation procedure, in terms of patients rather than individual specimens, is given in Table IV. In 19 patients, comprising 4 per cent of the total study group (or 11 per cent of the 188 individuals yielding a virus), the agent was detected in suckling mice only. This group of 19 included all of the 13 patients yielding Coxsackie virus types A2 through A7 and 1 patient with an unidentified virus type, but accounted for only 5 of the 114 patients with Group B Coxsackie virus infections. The use of SM also contributed to the detection of a dual infection in 2 patients, as described above. In the remainder of the cases in which an enterovirus was recovered, the viruses were detected in both MK and SM (57 per cent) or in MK only (32 per cent). Thus, of the total group of enterovirus infections recognized, approximately 90 per cent were detected by means of MK and 65 per cent by means of SM.

Increase in viral isolations from testing multiple stool specimens. The receipt of multiple stool specimens from a number of patients in the study group made it possible to assess the added diagnostic value of testing more than a single specimen. The frequency of recovery of an enterovirus from a second or third specimen following an earlier negative specimen is shown in Table V. These data include only those cases in which 3 or more stools were submitted and an identified enterovirus was recovered from at least one specimen. The median intervals between onset of symptoms and collection of the specimens were 5, 7, and 9 days, and all specimens were collected within a range of 1 to 21 days. It may be seen that about 90 per cent of the infections with Groups A and B Coxsackie viruses and with poliovirus were detected through examination of the first specimen; in SM, 97 per cent of the Coxsackie B infections were detected with the first specimen. Among patients yielding ECHO viruses, however, about one-third of the initial specimens were negative, and there was a more decided gain from the testing of second and third specimens. Fourth specimens were tested from 37 of the same patients shown in Table V (and from a larger number of patients from whom no virus was recovered), but in no instance was an infection first detected by means of the fourth specimen. In a previous study of the recovery of poliovirus from serial stool specimens, the virus was first isolated from the initial specimen of 85 per cent of the patients, and rarely ($<$ 2 per cent) from fourth and fifth specimens.[8]

Coxsackie Group B infections missed by virus isolation attempts. In a group of 200 patients in the present study, neutralizing antibody levels to Group B Coxsackie viruses in acute- and convalescent-phase sera were determined[9] in addition to the attempted isolation of virus in both SM and tissue culture. In 95 cases, evidence of a current infection with a Group B Coxsackie virus was elicited by at least one of these procedures. The number of infections detected by each diagnostic procedure is shown in Table VI. The close parallel between the recovery of Group B viruses in tissue culture or SM is again apparent, with either method alone detecting just over 80 per cent of the total group. Among the 82 patients from whom a virus was recovered, a fourfold or greater rise in

the homotypic neutralizing antibody titer was demonstrated in 42. With 2 exceptions, those failing to manifest a significant rise had homotypic antibody titers of 1:32 or greater in the convalescent-phase specimen. Thirteen patients (14 per cent) whose stools failed to yield a virus in SM or tissue culture showed significant rises in antibody to one or more types of Coxsackie B viruses. These individuals are presumed to represent infections in which virus either was not present in the specimens tested or was missed in the isolation attempts. Thus diagnostic rises in antibody titer, i.e., fourfold or greater increases, could be demonstrated in 55 patients, or 58 per cent of the total group of infections recognized by the combined results of all 3 diagnostic procedures.

Comment

It is well established that Coxsackie Group B viruses are among the common causative agents of the aseptic meningitis syndrome[1] and there is appreciable evidence implicating these viruses in some cases of acute myelitic and encephalitic disease.[4, 10] At least 2 types of Coxsackie Group A viruses, A7 and A9, have been incriminated in cases of aseptic meningitis, and some strains of type A7 are known to cause paralytic poliomyelitis-like illness in man and monkeys.[10]

In addition, reports by several investigators[1] of the recovery of both poliovirus and various Group A Coxsackie viruses from patients with clinical poliomyelitis have suggested an unexplained natural tendency for these infections to occur concurrently. Dalldorf and Weigand[11] demonstrated experimentally that combined infection with certain Group A viruses and an attenuated poliovirus resulted in more severe disease than either virus alone was capable of causing.

Thus, at the outset of the present study, it appeared that the systematic use of SM in addition to tissue culture for the attempted recovery of enteroviruses from patients with acute central nervous system disease might detect an appreciable number of Coxsackie A viruses, both singly and in association with other enteroviruses. There was also interest in the possibility that the regular use of both host systems for the isolation of viruses might considerably enhance the recovery of Coxsackie Group B viruses and perhaps some strains of ECHO viruses, particularly since laboratory studies in California in previous years consistently had failed to demonstrate any etiologic agent in over one-third of the cases of aseptic meningitis. In fact, however, the net gain from the use of suckling mice for virus isolation attempts from the stools of 460 patients with acute central nervous system disease studied over 2 successive years proved to be rather meager. Thus, from 21 patients, approximately 10 per cent of those yielding an enterovirus, or less than 5 per cent of the entire study group, a virus was recovered in SM that was not detected in tissue culture.

It is to be expected that the predominant types of enteroviruses giving rise to central nervous system disease will vary from year to year and place to place. The relatively minor added contribution of SM in this study was found at a time when Coxsackie viruses B4 and B5 were strongly predominant and Group

A viruses apparently were not prevalent. In general, however, it appears reasonable, for laboratory diagnostic purposes in cases of central nervous system disease, to use suckling mice as an adjunct to tissue culture for testing only those specimens which fail to yield a virus in tissue culture rather than as a duplicate isolation procedure.

The authors gratefully acknowledge the generous assistance given in the collection of clinical specimens by Dr. Evelynne Knouf, Los Angeles County General Hospital; Dr. Herbert Cowper, Los Angeles City Health Department; and Dr. Mary Dale, Los Angeles County Health Department. Mr. Raymond Guenther of this laboratory gave valuable assistance in the collation and analysis of the data.

REFERENCES

1. Dalldorf, G., Melnick, J. L., and Curnen, E. C.: The Coxsackie Virus Group, *in* Rivers, T. M., and Horsfall, F. L., Jr., editors: Viral and Rickettsial Infections of Man, ed. 3, Philadelphia, 1959, J. B. Lippincott Company, pp. 519-546.
2. Robinson, C. R., Doane, F. W., and Rhodes, A. J.: Report of an Outbreak of Febrile Illness With Pharyngeal Lesions and Exanthem, Toronto, Summer 1957—Isolation of Group A Coxsackie Virus, Canad. M. A. J. **79**: 615, 1958.
3. Lennette, E. H., and Wiener, A.: Unpublished observations.
4. Lennette, E. H., Magoffin, R. L., Schmidt, N. J., and Hollister, A. C., Jr.: Viral Disease of the Central Nervous System. Influence of Poliomyelitis Vaccination on Etiology, J. A. M. A. **171**: 1456, 1959.
5. Schmidt, N. J., and Lennette, E. H.: An Inquiry Into the Use of the Complement Fixation Test for the Typing of Poliomyelitis Viruses, Am. J. Hyg. **66**: 119, 1957.
6. Schmidt, N. J., Midulla, M., and Lennette, E. H.: A Colorimetric Method for the Typing of Coxsackie Viruses of Group B and Certain Members of Group A, J. Immunol. **80**: 454, 1958.
7. Schmidt, N. J., and Lennette, E. H.: A Complement Fixation Test for Poliomyelitis, J. Exper. Med. **102**: 133, 1955.
8. Lennette, E. H.: Problems of the Viral Diagnostic Laboratory With Respect to Poliomyelitis, *in* Poliomyelitis: Papers and Discussions Presented at Fourth Internat. Poliomyelitis Conf., Philadelphia, 1958, J. B. Lippincott Company, pp. 377-386.
9. Lennette, E. H., Shinomoto, T., Schmidt, N. J., and Magoffin, R. L.: The Neutralizing Antibody Response to Coxsackie Group B Infections in Patients With CNS Disease, J. Immunol. **86**: 257, 1961.
10. Kibrick, S.: The Role of Coxsackie and ECHO Viruses in Human Disease, M. Clin. North America **43**: 1291, 1959.
11. Dalldorf, G., and Weigand, H.: Poliomyelitis as a Complex Infection, J. Exper. Med. **108**: 605, 1958.

Plasma protein antigens in the histopathologic vascular response to experimental sensitization

SHELDON G. COHEN, M.D.,* and THERESA M. SAPP, M.T.**
Wilkes-Barre, Pa.

Reversed passive subanaphylactic sensitization techniques were employed to effect lesions of pulmonary periarteritis and panarteritis, glomerulitis, and myocarditis in the rabbit. This experimental model of "serum sickness" was utilized in the study of antigenic relationships of the purified bovine plasma fractions, albumin, gamma globulin, and fibrinogen to the histopathogenesis of vascular sensitization. Identical lesions of vasculitis and glomerulitis resulted from in vivo immunologic reactions involving each of the 3 foreign plasma fractions suggesting their lack of specificity for a vascular tissue site of selective action. Application of this experimental technique further demonstrated an in vivo cross-reactivity of albumin, gamma globulin, and fibrinogen antigen-antibody systems with each other as well as for foreign species proteins (albumin with ovalbumin, and gamma globulin and fibrinogen with the respective corresponding human plasma fractions). This is explained by the finding of common components of significant antigenicity even in the range of trace impurities indicated in in vitro immunologic test procedures. Although considered chemically distinct these fractionated plasma proteins do not meet standards for homogeneity on the basis of their biologic activity.

In a previous report[1] it was noted that the experimental procedure of reversed passive subanaphylactic sensitization employing whole bovine serum was responsible for the development of lesions of (1) periarteritis and panarteritis, (2) pulmonary eosinophilic granulomas, (3) myocarditis, and (4) glomerulitis in the rabbit. Extention of the application of this technique was intended to demonstrate etiologic roles for individual purified bovine

From the Department of Biology, Wilkes College, Wilkes-Barre, Pa.
Supported in part by a research grant from the Northeastern Pennsylvania Heart Association, and by National Heart Institute Grant No. H-3890, the National Institutes of Health.
Received for publication Nov. 25, 1960.
*Associate Professor of Biological Research.
**Research Assistant.

plasma protein fractions in the histopathogenesis of each of these cardiovascular-renal alterations. However, study of this experimental model of "serum sickness" served to emphasize the importance of other factors concerned with the biologic activity of bovine plasma albumin, gamma globulin, and fibrinogen in evoking tissue responses through immunologic mechanisms. These included demonstrations of their lack of antigenic homogeneity, their cross-reactivity with heterologous species proteins, and the antigenic significance of trace components for each fraction.

Materials and methods

Experimental procedure. The experimental plan employing bovine plasma fractions* was similar to that described for passive reversed subanaphylactic sensitization techniques utilizing whole bovine serum or plasma and homologous species antiserum in rabbits.[1]

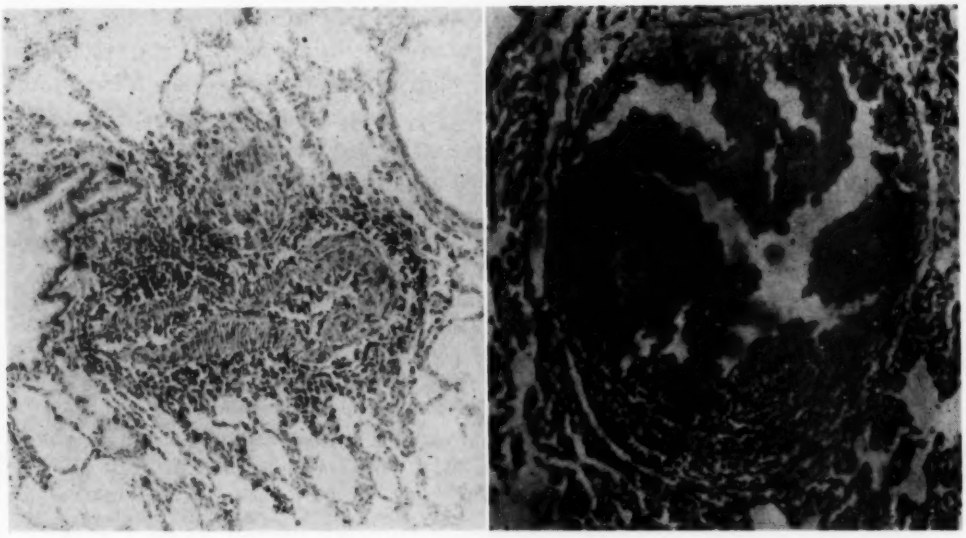
Antisera were prepared by rabbit immunizations to either crystallized bovine plasma albumin (BPA),† bovine gamma globulin (BGG),† or bovine plasma fibrinogen (BPF).† Each animal received initial injections totaling 500 mg. of the plasma fraction absorbed on aluminum hydroxide gel on the same day in both multiple subcutaneous and intramuscular sites. During the next 2 weeks 100 mg. was administered by 4 spaced injections, the initial 2 intravenously and the remaining 2 by subcutaneous routes. Seven days after the last injection blood was obtained from each animal by cardiac puncture; the serum was separated, and its antibody content was determined by standard immunochemical quantitative precipitin test techniques.

Forty-five normal and previously untreated rabbits ranging in weight from 2.5 to 3.0 kilograms composed the experimental series. Each received through the marginal ear vein, 350 mg. of a purified bovine plasma fraction dissolved in sterile saline. Twenty-four hours later a serum sample was obtained from blood withdrawn through the central artery of the ear. The presence of circulating antigen was demonstrated by interfacial serum precipitin tests with corresponding antiserum. The animal was then subjected immediately to reversed passive subacute anaphylactic challenge with homologous species antiserum. An amount of this antiserum containing 2 to 3 mg. of antibody nitrogen diluted in physiologic saline to a total volume of 50 ml. was administered by slow intravenous drip through the marginal ear vein at the rate of 20 to 25 drops per minute. Twenty-four hours later the rabbit was sacrificed by air embolism, and tissue sections from the lungs, heart, and kidneys were prepared for histologic study.

Ten rabbits received BPA, 10 BGG, and 10 BPF. Six of each 3 groups were challenged with corresponding antiserum. The remaining 12, in pairs, were challenged with antiserum to each of the unrelated purified bovine plasma fractions. Six rabbits each received equally divided doses of BPA, BGG, and BPF combined and the challenging dose of mixed antisera contained equal amounts of antibody nitrogen to all 3 plasma proteins. Nine rabbits were given a foreign species protein and then challenged with an antiserum to the related bovine plasma protein fraction, 3 receiving egg albumin (EA)‡ and anti-BPA serum; 3 human serum gamma globulin (HGG)‡ and anti-BGG serum; and 3 human plasma fibrinogen (HPF)‡ and anti-BPF serum.

Characterization of antigens and antisera. Agar-gel double diffusion precipitin tests employing the Ouchterlony plate technique were utilized to characterize qualitatively individual component antigen-antibody systems for each purified bovine plasma fraction

*The Armour Laboratories, Kankakee, Ill.
†BPA, BGG, and BPF were obtained from The Armour Laboratories, Kankakee, Ill.
‡EA obtained from Nutritional Biochemicals, Cleveland, Ohio; HGG from Parke, Davis & Company, Detroit, Mich.; and HPF from Merck Sharp & Dohme, West Point, Pa.



A. *B.*

Fig. 1. Lung sections illustrating vascular changes taken from rabbits subjected to reversed passive sensitization to bovine plasma fractions. *A*, BPA + anti-BPA serum; *B*, BPA + anti-BGG serum. The small pulmonary artery in *A* is involved with periadventitial cellular infiltration consisting chiefly of eosinophilic granular cells and some polymorphonuclear leukocytes. Eosinophils are also noted infiltrating through the media and intima. Edema of the media and disruption in the intimal cellular continuity are evident. In *B* there is thrombus formation and extensive eosinophilic cellular infiltration throughout the right lateral portion of the vessel wall.

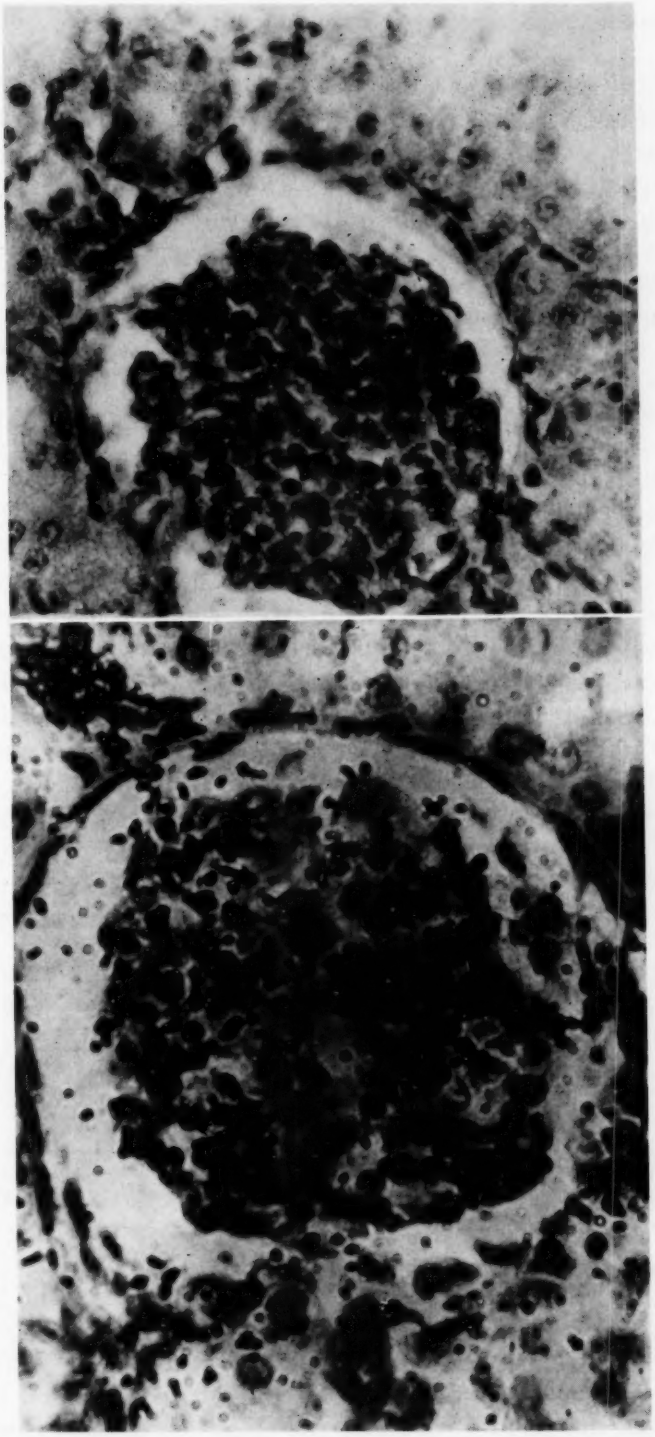
Table I. Bands representing reactions of identity developed between anti-BPA serum, anti-BGG serum, and anti-BPF serum when these were tested against whole bovine serum and plasma

Antigen	*No. of precipitin bands with antiserum to:*		
	BPA	*BGG*	*BPF*
Bovine plasma albumin	4	2	1
Bovine gamma globulin	2	4	2
Bovine plasma fibrinogen	3	2	6
Bovine serum	6	5	6
Egg albumin	2		
Human serum gamma globulin		1	
Human plasma fibrinogen			2

and to detect cross-reactive constituents among the unrelated purified bovine proteins and between the corresponding proteins of foreign species origin. The results of these tests are summarized in Table I.

Results

Pathologic findings. Where lesions were found in the lungs and kidneys they were typical of those noted and described in previously reported studies[1, 2] where passive sensitization techniques were employed using anti-



A.

B.

Fig. 2. Kidney sections taken from rabbits subjected to reversed passive sensitization to bovine plasma fractions. *A*, BGG and anti-BGG serum; *B*, BPA and anti-BGG serum. Typical glomerular involvement is demonstrated with cellular edema and hypercellularity. Red blood cells can be seen within the capsular space in *B*.

sera to whole foreign species sera. In brief these included the diseases described below.

A. ARTERITIS (FIG. 1). The small pulmonary arteries and arterioles were chiefly involved in segmental fashion. These exhibited varying degrees of perivascular and adventitial infiltrations chiefly with eosinophilic granular cells and with lesser numbers of polymorphonuclear leukocytes. Periadventitial leukocytes and eosinophils occasionally were observed in nodular granulomatous-like collections about portions of the affected vessel. Perivascular cellular infiltrates often extended into adjacent pulmonary parenchyma. Eosinophils were also seen infiltrating the intima and through the media. Edema, disruption in the morphologic continuity, and early proliferation of endothelial cells occurred within the intima. Intraluminal thrombus and cellular aggregate formation were frequently noted. Adventitial and medial edema were often evident and rarely were mural necrotic changes demonstrated.

B. GLOMERULITIS (FIG. 2). Renal lesions consisted of glomerular alterations characterized by leukocytic infiltrations and edema of basement membrane and endothelial cells with increased glomerular cellularity.

C. MYOCARDITIS. Myocardial lesions consisted of scattered collections of eosinophilic granular cells within the interstitial tissue. Occasionally, cellular infiltrations within focal areas of the right ventricular wall were associated with other inflammatory changes consisting of interstitial edema and necrosis of myocardial fibers.

Preliminary studies[1, 2] demonstrated that lesions of this type are not produced by either foreign serum constituents or their antisera alone but arise as a specific vascular tissue response to in vivo antigen-antibody interreaction.

Table II

Challenging antiserum to:	*Sensitizing antigen*	*No. of animals*	*No. exhibiting lesions of:*		
			Pulmonary arteritis	*Myocarditis*	*Glomerulitis*
Bovine plasma albumin	BPA	6	6	0	3
	BGG	2	2	1	2
	BPF	2	1	1	1
	EA	3	3	0	2
Bovine gamma globulin	BGG	6	6	1	3
	BPA	2	2	1	2
	BPF	2	2	1	2
	HGG	3	3	1	1
Bovine plasma fibrinogen	BPF	6	6	0	4
	BGG	2	2	0	0
	BPF	2	1	0	1
	HPF	3	3	1	1
B–PA+GG+PF	B–PA+GG+PF	6	6	1	4

The results of histopathologic study of the organs of each group of passively sensitized-challenged rabbits are summarized in Table II.

Discussion

The technique of reversed passive subanaphylactic sensitization applied to bovine plasma fraction antigen-antibody systems and patterned after experimental conditions employing whole bovine serum[1] has been found equally favorable for the development of vasculitis in the rabbit. Prior control studies[1, 2] had demonstrated that these histopathologic vascular alterations are dependent upon antigen-antibody union and do not result from the presence of either foreign serum antigens or from antibody per se. Although exact minimum requirements of reactants have not been established, the range of 350 mg. of antigen and 2 to 3 mg. antibody N has been found optimal in this procedure. Infrequently, the development of segmental arteritis or glomerulitis was noted following challenge with antisera containing as little as 100 to 300 μg of antibody N.

It was felt that advantages offered by passive sensitization techniques[1, 2] might be utilized in a study of relationships between variations in antigenic character and histopathogenesis of experimental serum sickness. This would allow for bypassing such uncontrolled in vivo variables usually associated with active sensitization as mechanisms of antibody production, mesenchymal reactions,[3] antigen clearance,[4] levels of antigen and antibody, their duration of effect, and the exact time of interreaction. Initial studies revealed that lesions of pulmonary periarteritis and panarteritis and of proliferative glomerulitis were identical in histopathologic detail and occurred in similar frequency in animals subjected to reversed passive subanaphylactic sensitization, regardless of the antigen-antibody system concerned. This was true of purified bovine plasma fractions, combination of the 3 (BPA, BGG, and BPF), or whole serum. Utilizing identical bovine plasma antigens employed by other investigators[4, 5] in active sensitization experiments we were unable to duplicate reported findings that suggested a correlation between the type and localization of "serum sickness" cardiovascular-renal lesions and the character of participating antigens. Interpretations related to variations in catabolic rate and duration of effect of individual proteins[4, 6] in the histopathogenesis of vasculitis have been favored over those related to possible selective influences of chemical properties of the antigens in question.

The characterization of components of the participating antigen-antibody systems may be additionally pertinent to this point. Agar-gel double diffusion precipitin tests (Table I) demonstrated the presence of multiple antibody systems in antiserum to each purified fraction, BPA, BGG, and BPF. Other evidence for immunologic heterogeneity of fractionated plasma proteins has been reported.[7-10] In vivo cross-reactivity was evident among BPA, BGG, and BPF antigen-antibody systems in effecting identical histopathologic lesions of vasculitis and glomerulitis (Table II). This was explained by the in vitro characterization of their antisera in precipitin tests with whole bovine serum (Table I) demonstrating cross-reactivity in vitro. The possibilities of

incomplete fractionation or the formation of complexes between distinct and unrelated constituents of normal serum[11] during fractionation procedures exist. However, the patterns of identity reactions in agar-gel further suggests the presence of trace quantities of impurities common to the 3 bovine plasma fractions with antigenicity enhanced to significant levels as a result of hyperimmunization procedures. Attention has been called to the importance of sensitization to minor quantities of antigenic impurities in other preparations, such as crystallized egg white[12] and diphtheria toxoid.[13]

Partial antigenic relationships between serum albumins and between serum globulins of different species origin has been reported.[10, 14-16] The antigenic significance of components common to BPA and EA, EGG and HGG, and BPF and HPF (Table I) is demonstrated by their degree of in vivo cross-reactivity sufficiently effective to evoke lesions of pulmonary panarteritis and periarteritis and of glomerulitis by passive sensitization procedures (Table II).

An etiologic role for BPA, BGG, and BPF in the experimental production of vascular-renal sensitization has been demonstrated by controlled techniques of passive sensitization. However, the development of identical lesions may arise on the basis of sensitization reactions to cross-reactive or to common antigenic components even in the range of trace impurities. Although considered chemically distinct, these fractionated plasma proteins do not meet critical biologic standards for homogeneity on the basis of their immunologic behavior. The interpretation of any experimental data suggesting selective localization for antigen and/or antibody in the histopathogenesis of vasculitis must therefore be open to question. This is especially true in the case of "serum sickness"–producing systems.

REFERENCES

1. Cohen, S. G., and Sapp, T. M.: Histopathologic Cardiovascular Responses to Passive Sensitization, J. Infect. Dis. **105**: 124, 1959.
2. Cohen, S. G., Mayer, L. D., and Criep, L. H.: The Experimental Production of Arteritis by Passive Sensitization, J. Immunol. **66**: 487, 1951.
3. Ehrich, W. E.: The Significance of Tissue Reactions Caused by Antigens, J. A. M. A. **135**: 94, 1947.
4. Germuth, F. G., Jr., Flanagan, C., and Montenegro, M. R.: The Relationships Between the Chemical Nature of the Antigen, Antigen Dosage, Rate of Antibody Synthesis and the Occurrence of Arteritis and Glomerulonephritis in Experimental Hypersensitivity, Bull. Johns Hopkins Hosp. **101**: 149, 1957.
5. Hawn, C. V. Z., and Janeway, C. A.: Histological and Serological Sequences in Experimenaal Hypersensitivity, J. Exper. Med. **85**: 571, 1947.
6. Germuth, F. G., Jr., and Heptinstall, R. H.: The Development of Arterial Lesions Following Prolonged Sensitization to Bovine Gamma Globulin, Bull. Johns Hopkins Hosp. **100**: 58, 1957.
7. Cohn, M., Deutsch, H. F., and Wetter, L. R.: Biophysical Studies on Blood Plasma Proteins; Analysis of Immunological Heterogeneity of Human Gamma Globulin Fractions, J. Immunol. **64**: 381, 1950.
8. Kabat, E. A., and Murray, A. R.: Comparison of Human Globulins in Their Reactivity With Rabbit Anti-Globulin by Quantitative Precipitin Method, J. Biol. Chem. **182**: 251, 1950.

9. Williams, A., Jr., and Grabar, P.: Immunoelectrophoretic Studies on Serum Proteins. I. The Antigens of Human Serum, J. Immunol. **74**: 158, 1955.
10. Dixon, F. J., and Maurer, P. H.: Specificity of the Secondary Response to Protein Antigens, J. Immunol. **74**: 418, 1955.
11. Williams, A. J., and Grabar, P.: Immunoelectrophoretic Studies on Serum Proteins. II. Human Gamma Globulins, J. Immunol. **74**: 404, 1955.
12. Vaughan, J. H., and Kabat, E. H.: Studies on the Antibodies in Rabbit Antisera Responsible for Sensitization of Human Skin. I. Role of Impurities in Crystalline Egg Albumin in Stimulating the Production of Skin-Sensitizing Antibody, J. Exper. Med. **97**: 821, 1953.
13. Finger, I., and Kabat, E. A.: A Comparison of Human Antisera to Purified Diphtheria Toxoid With Antisera to Other Purified Antigens by Quantitative Precipitin and Gel Diffusion Techniques, J. Exper. Med. **108**: 453, 1958.
14. Melcher, L. R., Masouredis, S. P., and Reed, R. J.: Immunological Relationship Between Human and Bovine Serum Albumin, J. Immunol. **70**: 125, 1953.
15. Maurer, P. H.: The Cross-Reactions Between Albumins of Different Species and Gamma Globulins of Different Species, J. Immunol. **72**: 119, 1954.
16. Weigle, W. O.: Immunochemical and Biological Properties of Cross-Reactive Systems, Fed. Proc. **19**: 205, 1960.

Experimental studies in the anemia of malignancy. I. The antiglobulin test in tumor-bearing hamsters

ANTHONY BETTS, M.D., PERRY G. RIGBY, M.D., CHARLES P. EMERSON, M.D., and GILBERT H. FRIEDELL, M.D. *Boston, Mass.*

Antiglobulin (Coombs) tests were performed on erythrocytes from tumor-bearing hamsters using rabbit anti-hamster-globulin serum. When the rabbit serum was inactivated by heating before absorption with normal hamster red cells the antiglobulin tests were negative. The use of serum that had not been inactivated by heating resulted in positive tests in 12 of 15 tumor-bearing animals, while results from all control animals were negative.

Ten of the 12 animals with positive antiglobulin tests were anemic, 2 were not. None of the 3 tumor-bearing animals with negative tests were anemic, suggesting that erythrocyte sensitization precedes the development of anemia.

In certain patients with cancer and anemia the Coombs antiglobulin test is positive, indicating the attachment of a globulin to erythrocyte membranes. Most of these instances have been in cases of malignant lymphomas, although Green and associates[1] reported a modified Coombs test to be positive in 43 per cent of 541 patients with various malignancies, compared to positive results of the same test in 15 per cent of 241 noncancerous patients. He also reported that "the red cells" from 50 per cent of rats bearing either transplanted or chemically induced tumors agglutinated with rabbit anti–rat-globulin serum,[1] but gave no details of the procedure.

Investigating blood changes in the tumor-bearing hamster, Sherman and colleagues[2] found that anemia was a relatively common complication. Further investigation by Sherman[3] and Friedell and co-authors[4] revealed that an indirect hemolytic factor in the tumor, probably acting through the reticuloendothelial system, was partially responsible for development of the anemia.

From the Departments of Pathology and Hematology, Massachusetts Memorial Hospitals, Boston, Mass.

Supported in part by Grant No. C-1959 from the National Cancer Institution, National Institutes of Health, United States Pulblic Health Service.

Received for publication Feb. 14, 1961.

Whether or not the erythrocytes of the tumor-bearing hamster were abnormal, however, was not known. Therefore, antiglobulin tests were performed on erythrocytes from tumor-bearing hamsters as a means of revealing one possible type of erythrocyte abnormality, namely, the attachment to the red cell membrane of some product of the tumor itself or of normal host tissues.

Materials and methods

Rabbit anti–hamster-globulin serum was prepared by a modified method of Emerson and co-workers.[5] Each of 3 rabbits of mixed stock was given a weekly subcutaneous injection of 1 milliliter of a 3:4 mixture of normal hamster serum and Freund's incomplete adjuvant* for 4 weeks. At the end of the fifth week the animals were bled and the serum was obtained. With a portion of this serum 6 successive absorptions with normal hamster erythrocytes were carried out at 4° C. over a 2 hour period, using equal volumes of serum and erythrocytes. After absorption, the agglutination titer of the rabbit serum for normal hamster erythrocytes in a 2 per cent suspension was 1:32/64.

During the first absorption of this portion of rabbit serum some hemolysis of the normal hamster erythrocytes was noted, upon which it was realized that through oversight the serum had not been inactivated by heating. The series of absorptions for this portion of serum was nevertheless carried through as outlined above. However, since the standard procedure for preparation of antiglobulin serum calls for inactivation of serum,[6] a second portion of rabbit serum was inactivated by heating at 56° C. for 30 minutes, then carried through the same series of absorptions.

Blood samples (0.5 ml.) were obtained from 10 golden hamsters bearing a transplantable hamster sarcoma (MCP). This tumor and others have been maintained in this laboratory by serial transplantation for several years. Hemoglobin values were determined by the usual hematologic techniques. The samples were then given as "unknowns" to the individual performing the antiglobulin tests. Duplicate tests were performed using serial dilutions of both the heated (inactivated) and the unheated (not inactivated) portions from the first lot of serum.

One-tenth milliliter of a 2 per cent saline suspension of thrice-washed red cells from each unknown sample was incubated at 37° C. with an equal volume of test serum for 30 minutes. A second tube contained the red cell suspension and an equal volume of unheated (not inactivated) serum. Saline and albumin controls were run in each instance. The presence of absence of agglutination was determined microscopically after centrifugation of the mixture for 2 minutes at 2,000 r.p.m.

The above procedure was then repeated uring erythrocytes from 5 hamsters bearing a transplantable melanoma (MM2) and 5 additional nontumor-bearing hamsters. Serum for these tests was obtained from the same rabbits after they had been given a fifth subcutaneous injection of the hamster serum–Freund's adjuvant mixture. This serum was also divided into 2 portions, one of which was heated to 56° C. for 30 minutes while the other was not heated. Both portions of serum were then absorbed with normal hamster red cells and the antiglobulin tests performed. After absorption the agglutination titer of both heated and unheated serum for normal hamster erythrocytes was 1:32/64.

Results

Results are shown in Table I. Eight of the 10 sarcoma-bearing hamsters and 4 of the 5 melanoma-bearing animals had positive antiglobulin tests when unheated (not inactivated) serum was used. Antiglobulin tests in all

*Difco Laboratories, Detroit, Mich.

Table I. Agglutination titers of heated and unheated rabbit anti–hamster-globulin serum against erythrocytes from tumor-bearing and normal hamsters

Hamster		*Heated serum agglutinating titer*	*Unheated serum agglutinating titer*	*Hemoglobin (normal 16 Gm. %)*
Sarcoma	1	1:32	1:1,024	11
	2	1:64	1:2,048	13
	3	1:32	1:1,024	13
	4	1:32	1:512	14.5
	5	1:64	1:64	16.5
	6	1:64	1:1,024	12
	7	1:32	1:2,048	9
	8	1:64	1:64	16
	9	1:32	1:512	12
	10	1:32	1:1,024	12
Melanoma	1	1:32/64	1:256	13.5
	2	1:32	1:32	16
	3	1:32	1:256	13.5
	4	1:32/64	1:256	16
	5	1:32/64	1:256	16
Normal	1-15	1:32/64	1:32	16

of these 12 animals when heated (inactivated) serum was employed were negative. Six of the 8 sarcoma-bearing hamsters with positive antiglobulin tests were anemic, as were 4 of the 5 melanoma-bearing animals.

Antiglobulin tests on erythrocytes from 5 melanoma-bearing animals were then repeated as above except that the previously active (not heated) serum was heated to 56° C. for 30 minutes. No positive tests were obtained with this serum.

Discussion

The antiglobulin test in tumor-bearing hamsters was positive only when the test serum was not inactivated by heating. In general, our results are similar to those reported by Green and associates.[1] Unfortunately for the sake of comparison, however, he does not state at what titer his sera agglutinated the normal rat red cells. Moreover, his definition of a positive test included a time factor which we did not employ.

The presence of positive antiglobulin tests in some tumor-bearing hamsters with normal hemoglobin values suggests that the appearance of the positive antiglobulin test precedes the development of the frequently present anemia, and that the latter may be a consequence of destruction of these abnormal red cells.

The results of these antiglobulin tests differ from those reported in studies of human cancer patients with anemia, in whom usually a negative direct antiglobulin test is found.[7, 8] This difference, however, may be the result of the difference in the technique used, namely, the use of heat-inactivated serum in Coombs standard human antiglobulin test.

REFERENCES

1. Green, H. N., Wakefield, J., and Littlewood, G.: The Nature of Cancer Anemia and Its Bearing on the Immunological Theory of Cancer, Brit. M. J. **2**: 779, 1957.
2. Sherman, J. D., and Patt, D. I.: Blood Cell Factors and Tumor Growth in the Cheek Pouch of the Golden Hamster (Mesocricetus Auratus), Cancer Res. **16**: 394, 1956.
3. Sherman, J. D.: The Relationship of Tumor Necrosis to Red Blood Cell Changes in the Hamster, Blood **14**: 1223, 1959.
4. Friedell, G. H., Sherman, J. D., and Sommers, S. C.: Spleen and Liver in the Anemia of the Tumor Bearing Hamster, A. M. A. Arch. Path. **70**: 363, 1960.
5. Emerson, C. P., Jr., Franklin, W., and Lowell, F. C.: Production of Potent Anti-Human-Globluin (Coombs Reagent) in Rabbits Immunized With Serum Adjuvant Mixtures, J. Immunol. **66**: 323, 1951.
6. Dunsford, I., and Grant, J.: The Anti-Globulin (Coombs) Test, Springfield, Illinois, 1960, Charles C Thomas, Publisher.
7. Shen, S. C., and Homburger, F.: The Anemia of Cancer Patients and Its Relation to Metastases to the Bone Marrow, J. LAB. & CIN. MED. **37**: 182, 1951.
8. Miller, A., Chodos, R. B., Emerson, C. P., and Ross, J. F.: Studies of the Anemia and Iron Metabolism in Cancer, J. Clin. Invest. **35**: 1248, 1956.

Laboratory methods

The use of radioisotopes to measure body fluid constituents. I. Plasma sulfate

E. MILLER, M.S., C. J. HLAD, JR., M.S., STANLEY LEVINE, J. H. HOLMES, M.D., and H. ELRICK, M.D. *Denver, Colo.*

The study of sulfate metabolism has been handicapped by the fact that current methods for the determination of plasma sulfate are either difficult or unreliable or require too large a sample for routine or repeated determinations. In this paper a new method for measuring plasma sulfate is described. The procedure is simple, accurate and reproducible, and requires only a small sample. It is based on the use of a barium solution containing radioactive barium to precipitate the sulfate.

The mean plasma sulfate level in 88 normal young adults was found to be 0.645 ± 0.17 mEq. per liter. No significant sex difference was found. Patients with renal insufficiency were found to have significantly elevated plasma sulfate levels which were decreased following dialysis on the artificial kidney. The results obtained in both normal subjects and patients with renal disease agree well with those obtained by previous methods for sulfate determinations.

The measurement of certain body fluid constituents normally present in small concentration often presents technical problems which make difficult or impractical a study of their behavior in health and disease. Plasma inorganic sulfate is an example of such a substance. Current methods for its determination are unsatisfactory for various reasons. The methods of Loeb and Benedict[1] and of Kleeman and colleagues[2] are too difficult and time consuming for routine use. The errors associated with the methods of Medes and Stavers[3] are too large for certain types of studies. The methods of Denis[4] and Loeb and Benedict[1] require too large a blood sample for routine or repeated determinations.

We have developed a new method for measuring plasma sulfate. This method is simple, accurate, and reproducible, and requires a small plasma sample. This

From the Radioisotope Service, Veterans Administration Hospital, and the Departments of Medicine and Pharmacology, University of Colorado School of Medicine, Denver, Colo.
Received for publication Oct. 20, 1960.

paper presents details of the method and results of its application in normal individuals as well as in patients with renal disease.

Materials and methods

The method is based upon the quantitative precipitation of plasma sulfate by a barium solution containing radioactive barium (Ba^{133}).

Rationale. Barium was selected because it forms a highly insoluble compound with sulfate (0.23 mg. per 100 ml. H_2O at 18° C.). Ba^{133} was chosen because it has a long half-life (7.2 years) and because it has gamma emissions of 0.057, 0.080, 0.3, and 0.357 mev., which can be measured in our scintillation well counter with an efficiency of 64 per cent.

Uranium acetate was used in preference to other protein-precipitating agents because it precipitates inorganic phosphate as well as proteins.[5] It is necessary to remove the phosphate because with it barium also forms an insoluble compound which would result in erroneously high sulfate determinations. Although the counting rate of the uranium acetate solution is about 1.4 per cent that of the barium chloride solution, the activity of the uranium acetate in the plasma supernatant is reduced by precipitation to approximately 0.02 per cent of that in the barium chloride solution.

Solutions. The 3 solutions used are as follows: Uranium acetate* $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$, 8.0 Gm. per liter (filtered); Barium chloride,† $BaCl_2 \cdot 2H_2O$, 2.5 mEq. per liter, specific activity 0.12 mc. per gram Ba; and Barium chloride,† $BaCl_2 \cdot 2H_2O$, 5.0 mEq. per liter, specific activity, 0.06 mc. per gram Ba.

Procedure. A protein, phosphate-free filtrate is obtained by adding a measured volume (V_u) of the uranium acetate solution to a measured volume (V_p) of plasma, mixing, and centrifuging. A measured volume (V_s) of supernatant is removed, and to this is added a measured volume (V_b) of a standard barium chloride solution of known concentration (M_b), resulting in barium sulfate precipitation. This precipitation is initiated at room temperature and continued for at least 4 hours at 5° C. The solution is then centrifuged and a sample of the supernatant solution is removed for radioactive assay, yielding the counting rate (C) of the unprecipitated barium fraction. The counting rate (C_o) of the standard barium chloride solution used for the precipitation is also obtained. All samples were pipetted into glass vials (Kimble, No. 60,900) and assayed for radioactivity in a Curtis Scientific scintillation well counter (2 by 2 inches crystal).

The concentration (M_s) of sulfate in the plasma sample is calculated by the equation:

$$M_s = \frac{C_o V_b - C(V_s + V_b)}{C_o\ V_b} \cdot \frac{(V_p + V_u)\ V_b}{V_s\ V_p} \cdot M_b, \qquad (1)$$

where concentrations (M) are given in milliequivalents per liter, volumes (V) in milliliters, and counting rates (C) in counts per second per milliliter.

In normal plasma, the following volumes were used: $V_p = 2$ ml., $V_u = 4$ ml., $V_s = 2$ ml., and $V_b = 1$ ml. (specific activity, 0.12 mc. per gram.)

Under these conditions, equation (1) reduces to:

$$M_s = 1.5 \frac{C_o - 3C}{C_o} M_b. \qquad (2)$$

Subjects. The normal plasma sulfate studies were made on healthy male medical students and healthy female nursing students. The abnormal plasma sulfate values were obtained from patients with acute and chronic renal insufficiency before and after artificial kidney dialysis.

Results

The efficiency of removal of inorganic phosphate from plasma by uranium acetate was studied by the use of P^{32}-labeled sodium phosphate. One milliliter of

*Baker CP grade, J. T. Baker Chemical Co., New York, N. Y.
†Mallinckrodt Reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.

Table I. Plasma phosphate (P^{32}) removal

Sample No.	*Sulfate (mEq./L.)*	*Minimum phosphate (P^{32}) removed (%)*
1	0.3	99.7
2	0.6	99.5
3	1.8	99.4
4	1.9	99.4
5	2.0	99.3
6	2.3	99.5
7	2.4	99.4
8	2.4	99.5
9	4.1	99.3
10	4.2	99.3
11	4.4	99.1
12	5.5	99.1
13	11.6	99.4

a P^{32} solution was added to 1 ml. plasma and followed by 2 ml. of uranium acetate solution. Samples of the P^{32} solution, the uranium acetate solution, and the filtrate obtained from the above were counted with a thin window Geiger counter. The counting rate of the uranium acetate was approximately 1 per cent of that of the P^{32} added. Table I contains the results of 13 such experiments. The sulfate values of the samples have a range of 0.3 to 11.6 mEq. per liter

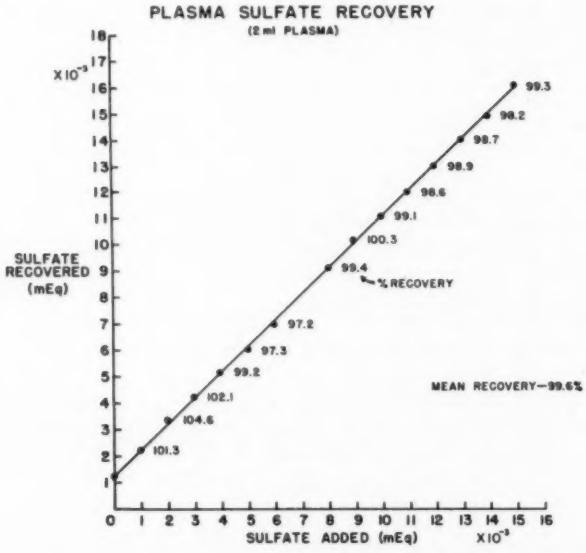


Fig. 1. The ordinate represents the amount of sulfate recovered by the isotope method described in the text. The abscissa represents the amount of sulfate added to each 2 ml. of plasma sample. The amounts are expressed in mEq. × 10^{-3}.

Table II. Plasma sulfate reproducibility of determinations

Series I mEq./L.	*Series II mEq./L.*	*Series III mEq./L.*
0.47	0.94	3.97
0.39	0.91	3.99
0.47	0.89	3.81
0.47	0.89	3.84
0.45	0.92	3.86
0.46	0.92	4.03
0.49	0.94	3.91
		3.99
		3.86
		3.93
Mean 0.457	0.916	3.92
Standard deviation 0.032	0.021	0.075

plasma. In all cases, the P^{32} activity remaining after precipitation was less than 1 per cent of that of the added P^{32}, which indicates that more than 99 per cent of the phosphate was removed.

Fig. 1 shows the results of a recovery experiment. Equal volumes of N/50 sulfuric acid (Fisher Reference standard solution) and N/50 sodium hydroxide were used with 2 ml. of plasma to give added amounts of sulfate from 1 to 15 mEq. per liter of solution. The mean recovery was 99.6 per cent, with a standard deviation of 1.9 per cent. A second recovery study of this type on 9 samples gave similar results. The combined mean recovery on 23 samples was 100 per cent, with a standard deviation of 1.9 per cent.

Table II contains data on the reproducibility of the method. The 7 determinations in series I were obtained on a plasma of low sulfate concentration. The mean is 0.457 ± 0.032 mEq. per liter with a range of 0.39 to 0.49. The values in series II were obtained on pooled plasma from hypertensive patients, and the mean is 0.916 ± .021 mEq. per liter, with a range of 0.89 to 0.94.* The 10 values in series III were obtained on pooled plasma from patients with renal disease and have a mean of 3.92 ± 0.075 mEq. per liter, with a range of 3.81 to 4.03 mEq. per liter.

The results of determinations on 88 normal subjects are shown in Table III. The mean plasma sulfate for 43 women is 0.64 ± 0.20 mEq. per liter; for 45 men is 0.65 ± 0.14. There is no significant difference between these mean values. The combined mean value for normal subjects is 0.645 ± 0.17 mEq. per liter.

Discussion

The method of choice for studying plasma sulfate levels should be simple, have a minimum error, and require a small sample of plasma. Denis,[4] using 10 ml. plasma samples and a nephelometric method, reported maximum errors of 5

*The cause for the elevated values of plasma sulfate in patients with hypertension is currently under study.

Table III. Normal plasma sulfate values

Subjects			
Female		*Male*	
Subject	*mEq./L.*	*Subject*	*mEq./L.*
J. C.	0.67	C. F.	0.56
N. A.	0.76	E. F.	1.35
B. B.	0.63	R. Ge.	0.75
J. B.	0.63	R. Go.	0.85
S. R.	0.61	D. G.	0.74
M. S.	0.63	G. G.	0.55
E. G.	0.61	D. H.	0.83
K. G.	0.47	S. G.	0.54
A. M.	0.62	J. Ha.	0.52
Y. M.	0.53	S. H.	0.59
A. S.	0.52	L. A.	0.75
P. S.	0.68	H. B.	0.78
L. T.	0.62	R. Bo.	0.77
P. T.	0.62	R. Ba.	0.62
P. U.	0.55	R. Be.	0.57
P. V.	0.61	J. B.	0.63
F. W.	0.71	C. Bo.	0.67
K. W.	0.65	C. Br.	0.65
J. W.	0.55	J. B.	0.72
J. M.	0.58	J. Hi.	0.57
E. N.	0.52	E. H.	0.49
E. O.	0.91	R. Ho.	0.48
M. O.	0.64	R. Hu.	0.56
C. P.	0.52	P. I.	0.62
P. P.	0.54	R. J.	0.49
S. P.	0.85	P. M.	0.57
C. P.	0.53	J. C.	0.49
B. R.	1.77	E. V.	0.62
K. R.	0.50	G. W.	0.52
O. S.	0.47	M. S.	0.63
S. S.	0.47	D. B.	0.63
K. S.	0.62	A. S.	0.59
L. S.	0.65	H. B.	0.63
N. W.	0.60	H. C.	0.70
S. W.	0.69	C. C.	0.68
V. V.	0.68	J. C.	0.63
J. S.	0.54	R. C.	0.59
A. M.	0.49	A. C.	0.54
N. S.	0.59	W. D.	0.69
S. A.	0.57	G. D.	0.78
J. D.	0.73	L. D.	0.76
W. C.	0.67	D. D.	0.62
		C. D.	0.52
		C. E.	0.74
		B. F.	0.71
Mean	0.64	Mean	0.65
Standard deviation	0.20	Standard deviation	0.14
Combined mean	0.645		
Standard deviation	0.17		

per cent. Loeb and Benedict[1] obtained good results by a gravimetric method. However, their procedure is difficult and requires 10 ml. of serum. The method of Medes and Stavers[3] has errors of recovery from –10 to +12 per cent. The method of Strickland and Maloney,[5] using flame spectrophotometry, requires 3 ml. of serum. The errors of this procedure appear minimal but the authors did not report enough data to establish its validity. Kleeman and associates[2] obtained good results using a benzidine sulfate colorimetric method. Their technique, however, requires considerable time and care in the preparation of solutions and the conduct of the procedure.

Our radioisotope procedure for measuring plasma sulfate has been shown to be simple and can be done on as little as 1 ml. of plasma. Recovery of added sulfate in plasma is complete and the reproducibility of the method is good over a wide range of plasma sulfate levels.

The normal values (0.65 ± 0.17 mEq. per liter) obtained by our method are in excellent agreement with those obtained by Kleeman and associates[2] and by Loeb and Benedict.[1] The former authors reported a mean of 0.59 mEq. per liter and a range of 0.46 to 0.81 mEq. per liter in 15 normal subjects; the latter authors reported a mean of 0.7 mEq. per liter and a range of 0.4 to 1.0 mEq. per liter in 13 normal subjects. The elevated plasma sulfate values obtained in patients with renal failure by the present method[6] are also in good agreement with those reported by Loeb and Benedict.[1]

An additional application of this method has been in the study of plasma sulfate values in patients in renal insufficiency, before and after dialyses with the artificial kidney. These results are reported in detail elsewhere.[6]

REFERENCES

1. Loeb, R., and Benedict, E.: Inorganic Sulfates in Human Blood, J. Clin. Invest. 4: 33, 1927.
2. Kleeman, C., Taborsky, E., and Epstein, F.: Improved Method for Determination of Inorganic Sulfate in Biological Fluids, Proc. Soc. Exper. Biol. & Med. 91: 480, 1956.
3. Medes, G., and Stavers, E.: Photelometric Determination of Inorganic Sulfate in Biological Fluids, J. LAB. & CLIN. MED. 25: 624, 1939.
4. Denis, W.: Sulfates in Blood, J. Biol. Chem. 49: 311, 1921.
5. Strickland, R. D., and Maloney, C. M.: Indirect Method for Determination of Serum Inorganic Sulfate by Flame Spectrophotometry, Am. J. Clin. Path. 24: 1100, 1954.
6. Holmes, J. H., Miller, E. S., and Hlad, C. J., Jr.: Serum and Urine Sulfate Changes in Uremia, Tr. Soc. Art. Int. Organs 6: 163, 1960.

Determination of phenformin in biologic fluids and tissues

LOUIS FREEDMAN, PH.D., MILTON BLITZ, B.S., EPHRAIM GUNSBERG, B.S., and SYLVIA ZAK, B.S. *Yonkers, N. Y.*

Methods for the determination of phenformin in biologic fluids and tissues are described. In plasma, the procedure consists of protein precipitation, extraction with chloroform-methanol from alkaline solution, acidification and evaporation of the solvent phase, reconstitution with water to specific volume, and color development with alphanaphthol-diacetyl reagent. In liver, kidney, or muscle tissues, the procedure consists of protein precipitation of 20 per cent sodium chloride tissue homogenates, extraction of the alkalinized salt solution with chloroform-methanol, adsorption from solvent on Folin-Decalso, elution with 5 per cent sodium chloride, and final color development. In separating phenformin from urine, the protein precipitation and extraction steps are applied as described for plasma. However, reconstitution is made with dilute alkali, which is then followed by adsorption from the alkaline solution on Decalso and elution with 1 per cent sodium chloride. Average recoveries and ranges (per cent) of added phenformin from plasma, tissues, and urine are 98.5 (95.6 to 100.5 per cent), 99.9 (97.5 to 104.0 per cent), and 96.2 (90.0 to 104.5 per cent), respectively. The procedure can detect and measure as little as 5 μ of phenformin and is applicable to metabolic and pharmacologic studies of this drug.

The development of phenformin ($N^1\beta$-phenethylbiguanide)* as an effective oral antidiabetic drug followed its reported pharmacologic effects in reducing blood sugar in various species of normal and hyperglycemic animals,[1-5] and its clinical evaluation in treatment of various types, including juvenile, of diabetes.[6-10] The drug has been investigated by many workers, and its mechanism of action in utilization of glucose has been partly elucidated.[11-15] A report of a symposium on chemical, pharmacologic, and clinical

From the Research Laboratories and the Division of Analytical Control, United States Vitamin and Pharmaceutical Corporation, Yonkers, N. Y.

Received for publication Feb. 15, 1961.

*DBI, a brand of phenformin, marketed by Arlington-Funk Laboratories, Division of United States Vitamin and Pharmaceutical Corp., Yonkers, N. Y.

investigations of the drug, under the auspices of the Baylor University Medical School and the Houston, Texas, Diabetic Association has been published.[16]

Materials and methods

Reports of metabolism of phenformin in animals or in man have been lacking, due primarily to lack of adequate procedures for determining small physiologic quantities of the drug in body tissues and in urine. A method for assaying phenformin to determine the "protein" binding affinity of purified plasma proteins for phenethylbiguanide in equilibrium dialysis studies was reported by Shepherd and McDonald.[17] This method, which covers a range of 80 to 200 μg of phenformin and a reaction time of 80 minutes at room temperature, is inadequate for determining the relatively small quantities, as little as 5 μg of phenformin, estimated to be present in certain body tissues and fluids after ingestion of the drug. A reliable method for this purpose is thus necessary to follow the metabolism of phenformin either as the free drug itself or as one or more of its degradation products.

The simple spectrophotometric and colorimetric methods used to determine phenformin in pharmaceuticals could not be applied directly to biologic fluids and tissues because of interferences from nitrogenous substances such as arginine · HCl, guanidine · HCl, methyl guanidine sulfate, creatine, and creatinine which normally are present. By various separation techniques and modifications the methods were made applicable to fluids and tissue.

Phenformin was determined in plasma by effectively separating it from the above interfering substances by treatment of the plasma with trichloracetic acid and extraction from an alkaline solution with chloroform-methanol mixture. Using this method, 95.6 to 100.5 per cent recoveries of added increments to control plasma were obtained.

When applied to tissues such as liver, kidney, and muscle, this procedure gave only 70 to 75 per cent recovery. However, homogenization of the tissue with 20 per cent sodium chloride solution and treatment with Folin-Decalso gave recoveries of 97.5 to 104 per cent.

Since urine contains relatively large amounts of interfering guanidine-like compounds, the methods applicable to blood and tissue could not be used. However, by elution of the final extract with more dilute sodium chloride solution than that used in the tissue procedure, namely 1 instead of 5 per cent, phenformin was quantitatively recovered from the Decalso column. This method gave excellent recovery, averaging 96 per cent.

Details of the various procedures developed are given below.

Determination of phenformin in plasma, tissue, and urine.

REAGENTS AND EQUIPMENT. (1) Trichloroactetic solution, 50 per cent (weight per volume).

(2) A, Sodium hydroxide solution, 10 N. B, Sodium hydroxide solution, 3 N. C, Sodium hydoxide solution, .02 N. (for urine only).

(3) Chloroform–absolute methanol solution, 85 per cent (volume per volume).

(4) Acidic ethanol solution : 2 N hydrochloric acid in 95 per cent ethanol (for plasma and urine only).

(5) Alphanaphthol-diacetyl developing reagent: 1.25 ml. 1 per cent diacetyl in n-propanol, plus 10 ml. 25 per cent alphanaphthol in n-propanol diluted to 50 ml. with n-propanol.

(6) Celite, filter aid, analytical grade.*

(7) Phenformin standard solution, 10 μg per milliliter, in distilled water.

(8) Ethanol, 95 per cent (for tissue and urine only).

(9) A, Sodium chloride, 20 per cent (for tissue and urine only). B, Sodium chloride, 5 per cent (for tissue only). C, Sodium chloride, 1 per cent (for urine only).

(10) Decalso, Folin† (for tissue and urine only). Purify Decalso as follows: Place a convenient quantity (100 to 200 Gm.) of Decalso in a 1,000 ml. Erlenmeyer flask, add

*Celite obtained from Johns Manville Co., New York, N. Y.
†Folin Decalso (Zeolite) obtained from the Permutit Co., New York, N. Y.

500 ml. distilled water, swirl, allow to settle, and decant the supernatant liquid. Repeat this treatment with 200 ml. 95 per cent ethanol, 200 ml. chloroform-methanol solution to which has been added 1 ml. trichloroacetic acid solution and 3 ml. 3 N sodium hydroxide, 2 more 200 ml. quantities of 95 per cent ethanol, and 50 ml. 20 per cent sodium chloride solution. Wash with sufficient distilled water until the last washing gives no reaction for chloride. Wash 3 more times with 200 ml. portions of 95 per cent ethanol and store the purified Decalso in 95 per cent ethanol.

(11) Base exchange tubes* (for tissue and urine only). Prepare the tube for use by placing a pledget of fine glass wool over upper end of capillary with the aid of a glass rod and a few milliliters of ethanol. Add an ethanolic suspension of purified Decalso to absorption tube, taking care to wash down all the silicate from walls of reservoir. Length of the Decalso column should be 12 cm. To keep air out of adsorption column (which may form air pockets in the adsorbent), a layer of liquid must be kept above the surface of the silicate throughout entire adsorption and elution process.

(12) Evelyn colorimeter equipped with a 565 mμ filter.

Determination of phenformin in plasma.

PREPARATION OF SAMPLES. Accurately pipette 3 to 5 ml. plasma into a 125 ml. glass-stoppered bottle, add 1 ml. trichloroacetic acid solution, mix, and allow to stand for 5 minutes. Add 50 ml. chloroform-methanol solution and 2 ml. 10 N sodium hydroxide solution and shake on a mechanical shaker for 20 minutes. Pour contents of bottle into a 25 by 150 mm. test tube, and centrifuge for 10 minutes. Aspirate and discard upper aqueous phase and precipitated mass. Pipette a 40 ml. aliquot of chloroform extract into a 50 ml. beaker and acidify immediately with 0.5 ml. acidic ethanol solution. Evaporate to dryness on a steam bath, immediately add exactly 5 ml. distilled water, and mix well.

PROCEDURE. Pipette an aliquot containing 10 to 30 μg into a 22 by 175 mm. test tube, dilute to 5 ml. with distilled water, add 1 ml. 3 N sodium hydroxide solution, and mix. Add 1 ml. developing reagent, mix well, and allow tube to stand undisturbed in the dark for 45 minutes at 37° C. After this period, shake tube well, add approximately 5 mg. Celite, mix, and filter through a small filter paper into an Evelyn colorimeter tube within 10 minutes of the time tubes are taken from the incubator. Measure absorbancy of the color developed in an Evelyn colorimeter with a 565 mμ filter and the tube holder in the 6 ml. position, setting the instrument at 100 per cent transmission with the reagent blank, prepared by adding the reagent to 5 ml. distilled water at the same time as the unknown sample.

PREPARATION OF STANDARD. Control the procedure with 1, 2, 3, and 4 ml. aliquots of the working standard solution, diluted to 5 ml. with distilled water in each tube, and proceed as described for sample.

CALCULATION. Prepare a standard curve by plotting absorbancies obtained on ordinate scale versus corresponding concentrations of phenformin used as the standard on abscissa scale. Calculate amount of phenformin in the unknown sample by determining concentration from the standard curve and multiplying by appropriate dilution factors.

Determination of phenformin in liver, kidney, and muscle tissue.

PREPARATION OF SAMPLE. Homogenize tissue with 20 per cent sodium chloride solution (approximately 300 to 350 mg. tissue per milliliter solution). Add a portion of the homogenate containing approximately 2 Gm. tissue to a 125 ml. glass-stoppered bottle, add 1.5 ml. trichloroacetic acid solution, shake virorously, and allow to stand for 5 minutes. Add 75 ml. chloroform-methanol solution and 3 ml. 10 N sodium hydroxide solution and shake on a mechanical shaker for 25 minutes. Pour contents of bottle into a 25 by 150 mm. test tube, and centrifuge for 10 minutes. Aspirate and discard upper aqueous phase and precipitated mass.

ADSORPTION AND ELUTION. Pass through the base exchange column two 15 ml. aliquots of the chloroform extract. Wash column with two 3 ml. portions of 95 per cent ethanol and discard the washings. Elute the phenformin with two 3 ml. portions of 5 per cent sodium chloride solution and 4 ml. water, and collect eluate in a 10 ml. volumetric flask. The entire

*Scientific Glass Apparatus Co., Catalog No. 1J-6035-S, Bloomfield, N. J.

chromatographic procedure is carried out with extreme caution to prevent the surface of liquid from falling below the surface of silicate until the final portion of sodium chloride solution has been added.

PROCEDURE, PREPARATION OF STANDARD, AND CALCULATIONS. Proceed as described for determination of phenformin in plasma.

Determination of phenformin in urine.

PREPARATION OF SAMPLE. Accurately pipette 4 ml. of urine into a 125 ml. glass-stoppered bottle, add 0.8 ml. trichloroacetic acid solution, mix, and allow to stand for 5 minutes. Add 50 ml. chloroform-methanol solution and 2 ml. 10 N sodium hydroxide solution and shake on a mechanical shaker for 20 minutes. Pour contents of bottle into a 25 by 150 mm. test tube and centrifuge for 10 minutes. Aspirate and discard upper aqueous phase. Pipette a 40 ml. aliquot of the chloroform extract into a 50 ml. beaker and acidify immediately with 0.5 ml. acidic ethanol solution. Evaporate to dryness on a steam bath, immediately add 5 ml. 0.02 N sodium hydroxide solution, and mix well.

ADSORPTION AND ELUTION. Pass 15 ml. 0.02 N sodium hydroxide solution through base exchange column. The column is now ready for use. Add the 5 ml. alkaline extract obtained on the column in the above section, "Preparation of Sample." Then add two 5 ml. rinsings of the beaker containing the alkaline extract. After this has percolated through, wash column with another 5 ml. distilled water. Elute with 3 separate 15 ml. portions of 1 per cent sodium chloride solution. Collect in a 50 ml. volumetric flask and dilute to mark. If phenformin concentration in the urine sample is low, use only one 15 ml. aliquot of the 1 per cent sodium chloride solution and collect without dilution. The entire procedure is carried out such that the silicate does not run dry until after final portion of sodium chloride solution has been added.

PROCEDURE, PREPARATION OF STANDARD, AND CALCULATION. Proceed as described under "Determination of Phenformin in Plasma."

Discussion

Methods are presented for determination of phenformin in various biologic materials. In pharmacologic and metabolic studies with phenformin, we determined the presence of the drug in liver and urine of animals by the above methods; as little as 5 μg has been detected. Indentification as phenformin has been confirmed by ultraviolet absorption and paper chromatography. We have not determined the presence of the drug in blood plasma and tissues other than the liver of animals fed the drug. The amount, if present, is apparently too small for these methods to detect. However, we were able to isolate as much as 20 μg per milliliter in blood plasma after subcutaneous injection in rabbits. It is hoped that the methods will form the basis for development of improvements which may allow quantities smaller than 5 μg to be isolated and detected and which will eventually lead to more complete knowledge of the mechanism of action of the drug.

REFERENCES

1. Ungar, G., Freedman, L., and Shapiro, S. L.: Pharmacological Studies of a New Hypoglycemic Agent, Proc. Soc. Exper. Biol. & Med. **95**: 190, 1957.
2. Ungar, G., Psychoyos, S., and Hall, H.: Action of Phenethylbiguanide, a Hypoglycemic Agent, on Tricarboxylic Cycle, Metabolism **9**: 36, 1960.
3. Williams, R. H., Tyberghein, J. M., Hyde, P. M., and Nielsen, R. L.: Studies Related to Hypoglycemic Action of Phenethylbiguanide, Metabolism **6**: 311, 1958.
4. Wick, A. N., Larson, E., and Serif, G. S.: A Site of Action of Phenethylbiguanide, a Hypoglycemic Compound, J. Biol. Chem. **233**: 296, 1958.

5. Freedman, L., Ungar, G., Shapiro, S. L., and Sadow, H. S.: Phenformin, Pharmacology, Toxicology and Clinical Use in Diabetes, Scientific Exhibit, A. M. A., Atlantic City, N. J.; June 8, 1959.
6. Pomeranze, J., Fujiy, H., and Mouratoff, G. T.: Clinical Report of a New Hypoglycemic Agent, Proc. Soc. Exper. Biol & Med. **95:** 193, 1957.
7. Krall, L. P., and Camerini-Davalos, R.: Early Clinical Evaluation of a New Oral Non-Sulfonylurea Hypoglycemic Agent, Proc. Soc. Exper. Biol. & Med. **95:** 345, 1957.
8. Krall, L. P., Bradley, R. F., and White, P.: Current Status of Oral Hypoglycemic Agents, J. Michigan M. Soc. **57:** 1422, 1958.
9. Krall, L. P., and Bradley, R. F.: Clinical Evaluation of Formamidinyliminourea, a New Biguanide Oral Sugar Lowering Compound: Comparison With Other Hypoglycemic Agents, Ann. Int. Med. **50:** 586, 1959.
10. Lambert, T. H.: Clinical Observations With a New Hypoglycemic Agent, Clin. Res. **6:** 91, 1958.
11. Tranquada, R., Kleeman, C., and Brown, J.: Acute Effects of DBI on Human Hepatic Intermediary Metabolism, Clin. Res. **7:** 110, 1959.
12. Pomeranze, J.: A New Oral Hypoglycemic Drug, New York J. Med. **58:** 3824, 1958.
13. Radding, R. S.: Clinical Experiences With New Oral Hypoglycemic Drugs in Diabetes, Texas J. Med. **55:** 110, 1959.
14. Krall, L. P., White, P., and Bradley, R. F.: Clinical Use of Biguanides and Their Role in Stabilizing Juvenile-Type Diabetes, Diabetes **7:** 468, 1958.
15. Tranquada, R. E., and Beigelman, P. H.: Metabolic Response of Rat Adipose Tissue to DBI, Clin. Res. **8:** 248, 1960.
16. Symposium on Phenformin: Baylor University College of Medicine, Houston, Texas, Feb. 5-6, 1959, Diabetes **9:** 163, 1960.
17. Shepherd, H. G., Jr., and McDonald, H. J.: Binding Affinity of Purified Plasma Proteins for Phenethylbiguanide, an Oral Hypoglycemic Compound, Clin. Chem. **4:** 496, 1958.

A colorimetric dry weight determination for tissue cultures and tissue homogenates

J. MARTYN BAILEY, PH.D., and A. MEYMANDI-NEJAD, B.SC.*
Washington, D. C.

A rapid colorimetric method for the determination of the dry weight of small samples of biologic materials is described. The method depends upon the determination of oxidizable carbon, by measuring the concentration of reduction products formed when the sample is heated with acid dichromate in aqueous solution. The procedure is particularly useful for measuring the dry weight of cultured cells or tissue homogenates from a variety of sources. It is suitable for analysis of from 0.2 to 2.0 mg. of material contained in up to 1 ml. of an aqueous suspension.

It is a frequent requirement during the culture of mammalian cells to obtain an accurate measure of the weight of tissue in a culture, both as a criterion of growth and as a measure by which to relate metabolic and other characteristics of the cells. One method which has been used to quantitate the growth of mammalian cells is to count the cells directly in some type of hemocytometer chamber.[1, 2] This method suffers from the disadvantage that most cells require some form of pretreatment, for example with trypsin or a complexing agent such as Versene, to disperse them in a form suitable for counting. Even when a suspension is obtained, cell clumping and disruption by the pretreatment render the method liable to error. Some of these disadvantages are overcome in the "replicate culture" method developed by Sanford and associates,[3] where the cells are pretreated with citric acid to strip the cytoplasm from the nuclei, and then the nuclei are stained and counted. The optimum conditions for the use of the counting method are met with cells, such as the MB III strain of mouse lymphoblasts, which grow as a single cell suspension.[1] It has been our experience, however, that even with these cells the precision of the method is

Supported in part by United States Public Health Service Research Grant No. H-5062 from the National Heart Institute.
Received for publication Dec. 29, 1960.
*Recipient of Lederle Laboratories Medical Student Fellowship.

rarely better than ± 10 per cent. A second and more significant disadvantage of counting methods from the point of view of metabolic determinations is that the weight of an individual cell may vary over a wide range, depending upon the conditions under which it is grown. For example, when the MB III strain of mouse lymphoblasts is grown under optimum conditions such that cell division is frequent, the dry weight averages about 0.22 mg. per million cells.[4] When the cells are cultured in a suboptimal growth medium such that cell division is less frequent, nuclear development often does not keep pace with cytoplasmic growth, and much larger cells result; one million of which may weigh as much as 1 mg. The errors which result, when metabolic or analytic results are based upon only cell counts in such situations, may be considerable.

The second class of methods for quantitating cell growth depends upon the determination by chemical means of some component of the cells, such as protein nitrogen[5] or nucleic acid phosphorus.[6] The determination may then be related to the dry weight of the tissue, if required, by the use of some predetermined conversion factor. These methods, although generally more accurate than the direct counting procedure, also suffer a major disadvantage in that the composition of cells may vary considerably when grown in different media.

We have sought, therefore, a method which would be more directly related to the dry weight of the cells without employing the impracticable procedure of drying and direct weighing of the small amounts of tissue involved in most laboratory tissue culture work (this approximates 1 mg. in an average roller tube culture). The method developed depends upon the determination of total carbon by heating with acid dichromate. The concentration of the reduction products is then measured colorimetrically. The procedure is similar to that developed by Bloor[8] for the determination of serum lipids, and is standardized

Table I. Conversion factors for dry weight determination

Test material	*Optical density per mg.*	*Conversion factor (O. D. to mg. dry weight)*
MB III strain cells	0.313	3.20
HeLa cells	0.257	3.90
L-strain cells	0.232	4.25
Rat muscle	0.232	4.32
Rat liver	0.223	4.48
Rat kidney	0.200	5.00
Rat intestinal mucosa	0.164	6.10
Liver microsomes	0.237	4.22
Liver mitochondria	0.206	4.86
Liver supernatant	0.178	5.62
Stearic acid	0.423	2.37
Crystalline albumin	0.212	4.72
Liver glycogen	0.162	6.18

Test samples were prepared as described in *Materials and methods.* All materials tested gave linear calibration curves in the range 0.2 to 2.0 mg. Conversion factors are calculated from the slope of the calibration curve over this range. They refer to the standard assay system described under "Procedure."

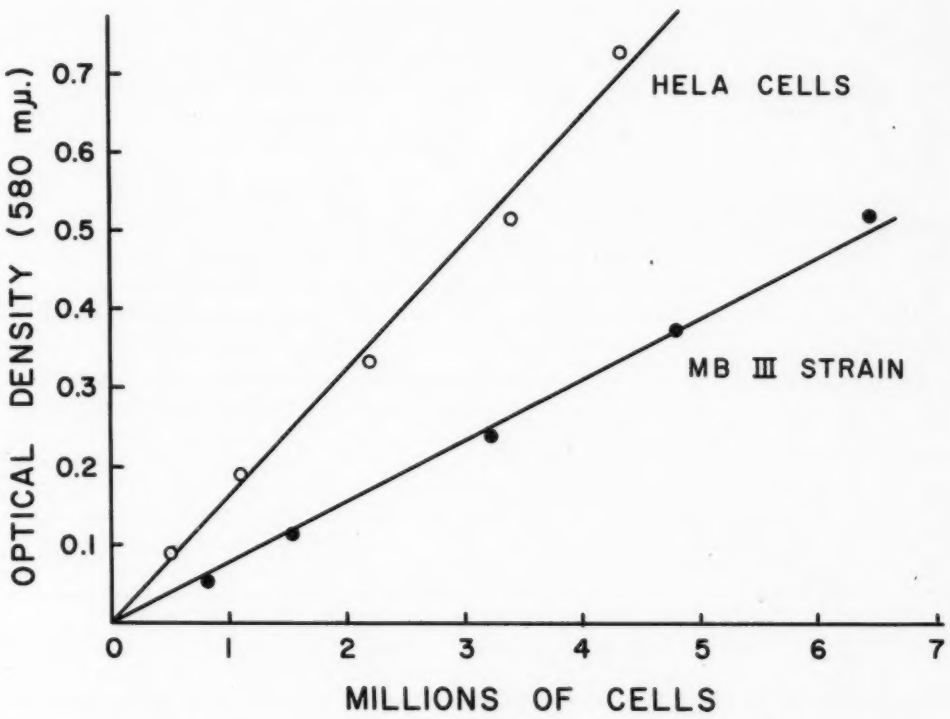


Fig. 1. Colorimetric determination of cell population in tissue cultures. Cells were grown in Blake bottles, as described in *Materials and methods,* and were washed twice with saline before counting and dispensing into tubes for the colorimetric determination.

for each cell type or tissue, by direct weighing of cells, or direct counting, if required. The method is also suitable for dry weight determinations of a number of mammalian tissues, tissue homogenates, and certain purified biologic materials. From 0.2 to 2 mg. of material is required.

Table II. Sensitivity of dry weight determination as a function of water content of test solution

Water added initially (ml.)	*Final color (580 mμ)*
0.0 (dry sample)	0.286
0.1	0.291
0.2	0.285
0.3	0.285
0.5	0.276
1.0	0.281
1.5	0.226
2.0	0.192
3.0	0.141

Each tube contained initially 1.3 mg. of crystalline albumin in 0.1 ml. of water. One tube was evaporated to dryness in the vacuum. Water was added to the remainder to give the volumes indicated, before adding the 2 ml. of dichromate reagent. After heating, each solution was made up to a final volume of 5 ml. with water before reading the color.

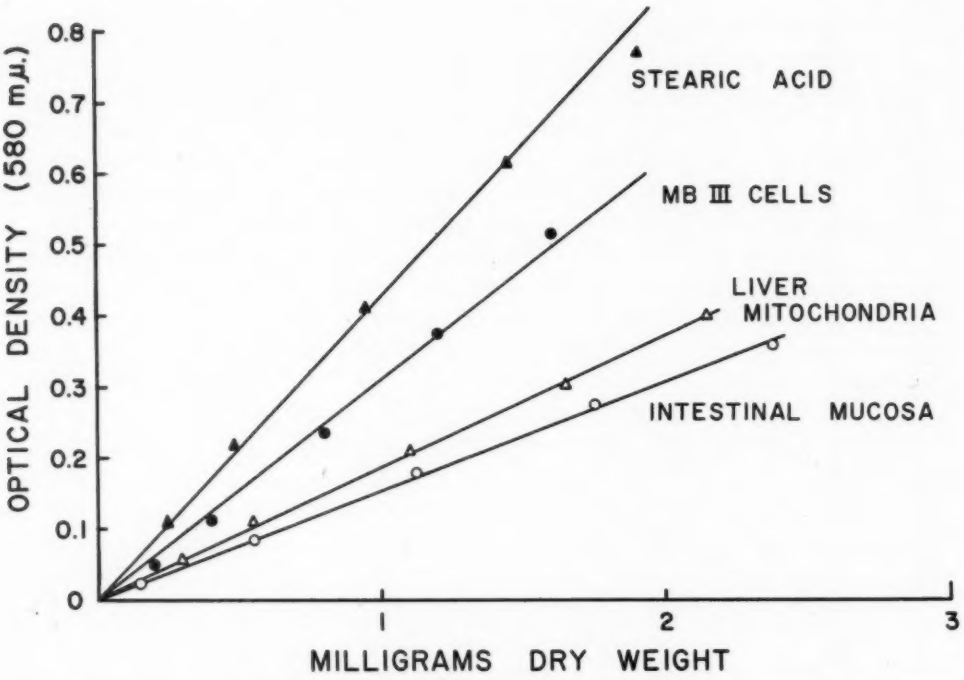


Fig. 2. Colorimetric dry weight determination of tissues. Samples were prepared for determination as described in *Materials and methods.* Stearic acid was dispensed in ether solution. The ether was then removed by heating for 30 minutes in a vacuum oven.

Materials and methods

Cells were grown in roller tube cultures,[9] or in large Blake bottles when larger quantities of material were required. The L-strain[10] of mouse fibroblasts was grown in a medium containing Evans-Earle N.C.T.C. 109[11] supplemented with 2 per cent human serum. The MB III strain of mouse lymphoblasts[12] was grown in 50 per cent human serum and 50 per cent balanced salt solution, as described previously.[13] HeLa cells[14] were grown in a medium consisting of 95 per cent Evans-Earle N.C.T.C. 109 supplemented with 5 per cent human serum. HeLa cells were dispersed by trypsinizing prior to determination of cell population and dry weight. Population determinations were made by counting aliquot portions of the cell suspensions in 0.2 by 0.0625 mm. Speirs-Levy hemocytometer chambers.

Homogenates of fresh rat muscle, intestinal mucosa, liver, and kidney were prepared by brief homogenization in isotonic saline using a Omnimixer.* Rat liver microsomes, mitochondria, and supernatant fractions were prepared from a homogenate of rat liver in isotonic saline, in the Spinco Model L preparative ultracentrifuge.[15] Crystalline bovine albumin was obtained from Nutritional Biochemicals Corporation, purified stearic acid from the California Foundation for Biochemical Research, and purified rat liver glycogen was prepared as described by Carrol and co-workers.[16] Other reagents and chemicals used were of analytic grade where available.

Cultured cells, harvested by centrifugation at 1,000 g, were washed twice with isotonic saline and suspended in 10 ml. of isotonic saline. A portion of the shaken suspension was removed for direct cell counting, and a second portion of 5 ml. was removed and evaporated to dryness at 110° C. for direct weighing. The weight was corrected for the salts in the

*Ivan Sorvall, Inc., Norwalk, Conn.

saline solution. The remainder of the suspension was dispensed into small test tubes (13 by 100 mm.) to give a range of approximately 0.2 to 2.0 mg. dry weight per tube, contained in not more than 1 ml. of solution. In our earlier experiments it was found that the determination was not affected if the material was present in up to 1 ml. of aqueous solution (see below). Samples were accordingly made up to 1 ml. with water before proceeding with the determination. A similar procedure was followed for the tissue homogenates and the purified compounds investigated.

Reagents: 2 per cent potassium dichromate in sulfuric acid. The reagent is prepared by adding a water-saturated solution containing 20 Gm. of reagent grade potassium dichromate slowly, with stirring, to 1 L. of reagent grade concentrated (36 N) sulfuric acid, so that the temperature does not rise above 100° C. The reagent is stored in a tightly stoppered glass bottle to exclude moisture, and is stable at room temperature for an indefinite period. Glassware used is cleaned by heating to 100° C. for 30 minutes in dichromate cleaning solution.

Procedure. The sample of material (0.2 to 2 mg. dry weight) contained in not more than 1 ml. of water or inorganic buffer is placed in a clean test tube (13 by 100 mm.), 2 ml. of the dichromate reagent is added, and the solution is heated on a boiling water bath for 30 minutes. The tube is removed and cooled for 3 to 5 minutes and water is added to give a final volume of 5 ml. The optical density of the solution is read at 580 mμ in the 1 cm. cells of the Beckman spectrophotometer against a reagent blank. This consists of 2 ml. of the dichromate reagent alone, subjected to a similar heating and dilution procedure. The color is stable for several hours. The dry weight of the unknown sample is calculated by comparison with a standard curve prepared by subjecting samples of known dry weight to the same procedure. Calibration curves of optical density plotted against millions of cells, or against milligrams dry weight of cells or tissue, are given in Figs. 1 and 2. Conversion factors for converting optical density to dry weight are collected in Table I.

Discussion

The time of heating with the dichromate reagent is not critical, and maximum color with most of the materials tested was usually developed after only 15 minutes' heating. The color was not changed by heating for an additional 1 hour. The 30 minute heating time, therefore, was chosen as a convenient interval. After heating and diluting to 5 ml. with water the color was stable for several hours. One disadvantage noted was that buffer salts with a high calcium content gave a cloudy suspension with the reagent and therefore should be avoided. Also, buffers or suspending fluids containing organic compounds must be excluded from the determination, if not, a correction should be applied. This is particularly true of organic solvents. We have used the method routinely for the determination of the dry weight of cultured cells after extracting the cellular lipids with ethanol-ether mixtures.[18] In order to obtain consistent results it was necessary to heat the cellular residue for 30 minutes at 110° C. to remove the last traces of solvents before proceeding with the determination. It was found that the determination was not affected by the presence in the sample of up to 1 ml. of water or aqueous saline. When more than 1 ml. of water was present, the sensitivity of the method was reduced (Table II). For samples containing more than 1 ml. of water, therefore, the excess was removed by prior evaporation in a vacuum oven.

The method provides a measure of oxidizable carbon in an organic sample since the colors given by crystalline albumin, glycogen, and stearic acid (Table I) are in approximately the same ratios as the carbon contents of these materials.

REFERENCES

1. Owens, Olga, von H., Gey, Margaret K., and Gey, George O.: Growth of Cells in Agitated Fluid Medium, Ann. New York Acad. Sc. **58**: 1039, 1954.
2. Puck, T. T., Marcus, P. I., and Cieciura, S. J.: Clonal Growth of Mammalian Cells In Vitro. Growth Characteristics of Colonies From Single HeLa Cells With and Without a "Feeder" Layer, J. Exper. Med. **103**: 273, 1956.
3. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E.: The Measurement of Proliferation in Tissue Cultures by Enumeration of Cell Nuclei, J. Nat. Cancer Inst. **11**: 773, 1951.
4. Bailey, J. M., Gey, Margaret K., and Gey, George O.: Carbohydrate Nutrition and Metabolism of a Strain of Mammalian Cells (The MB III Strain of Mouse Lymphoblasts) Growing In Vitro, J. Biol. Chem. **234**: 1042, 1959.
5. Oyama, V. I., and Eagle, H.: Measurement of Cell Growth in Tissue Culture With a Phenol Reagent (Folin-Ciocalteau), Proc. Soc. Exper. Biol. & Med. **91**: 305, 1956.
6. Siminovitch, L. A., Graham, A. F., Lesley, S. M., and Nevill, Ann: Propagation of L-Strain Mouse Cells in Suspension, Exper. Cell Res. **12**: 299, 1957.
7. Bailey, J. Martyn, and Gey, George O.: The Mechanism of Cholesterol Uptake by Cultured Mammalian Cells, Fed. Proc., vol. 18, 1959.
8. Bloor, W. R.: A Colorimetric Procedure for the Determination of Small Amounts of Fatty Acid, J. Biol. Chem. **170**: 671, 1947.
9. Gey, George O.: An Improved Technique for Massive Tissue Culture, Am. J. Cancer **17**: 752, 1933.
10. Sanford, K. K., Earle, W. R., and Likely, J. D.: The Growth In Vitro of Single Isolated Tissue Cells, J. Nat. Cancer Inst. **9**: 229, 1948.
11. Evans, V. J., Bryant, J. C., Fioramonti, F. C., Mcquilkin, W. T., Sanford, K. K., and Earle, W. R.: A Protein-Free, Chemically Defined Medium for Strain L-Cells, Cancer Res. **16**: 77, 1956.
12. De Bruyn, W. M., and Gey, George O.: Further Studies In Vitro of Transplantable Mouse Lymphosarcoma (MB TS6 157), Acta Unio internat. contra cancrum **7**: 772, 1952.
13. Bailey, J. Martyn, Gey, George O., and Gey, Margaret K.: Utilisation of Serum Lipids by Cultured Mammalian Cells, Proc. Soc. Exper. Biol. & Med. **100**: 686, 1959.
14. Scherer, W. F., Syverton, J. T., and Gey, George O.: Studies on Propagation In Vitro of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived From an Epidermoid Carcinoma of the Cervix, J. Exper. Med. **97**: 695, 1953.
15. Zamecnick, Paul C., and Keller, Elizabeth B.: Relation Between Phosphate Energy Donors and Incorporation of Labelled Amino Acids Into Proteins, J. Biol. Chem. **209**: 337, 1953.
16. Carrol, Nicholas V., Longley, Robert W., and Roe, Joseph H.: The Determination of Glycogen in Liver and Muscle by Use of Anthrone Reagent, J. Biol. Chem. **220**: 583, 1956.

Accurate! Reproducible! Sensitive!

COLORIMETRIC CHLORIDE DETERMINATION

in Serum, Plasma, Urine, Sweat, and Cerebro-Spinal Fluids with new

UNI-TECH CHLORI-PAK

COMPARE THESE ADVANTAGES AGAINST YOUR CURRENT PROCEDURE:

- Adaptable to all Colorimeters. Read at 520 mu.
- Requires only micro-quantities of fluid . . . as little as 0.01 ml. (Especially valuable when only small amounts are available).
- Sensitive to less than 10 gamma of chloride . . . important in sweat determinations.
- Total procedure completed in approximately 2 minutes.
- When series of chloride determinations is performed individual determinations take less than 60 seconds.

ELIMINATE THESE TIME-CONSUMING STEPS AND MANIPULATIONS:

- Standardization of Mercuric Nitrate Solution
- Titration
- Buretting
- Preparation of protein-free filtrate

NEW CHLORI-PAK, PLUS THESE OTHER UNCONDITIONALLY GUARANTEED KITS ARE AVAILABLE FROM YOUR DEALER

URI-PAK — Colorimetric Cyanide-Free Method for Uric Acid Determination

CHOLE-TECH — Colorimetric Total Cholesterol Determination.

ESTER-PAK — "One tube" Determination for Free Cholesterol and Cholesterol Esters.

LIPA-PAK — Serum Lipase Determination in 10 minutes.

AMYL-PAK — Colorimetric Serum and Urine Amylase Determination in 15 minutes.

TRANSAMINASE DUO KIT — Colorimetric Determination of SGO and SGP Transaminase.

Write For Free Procedures:

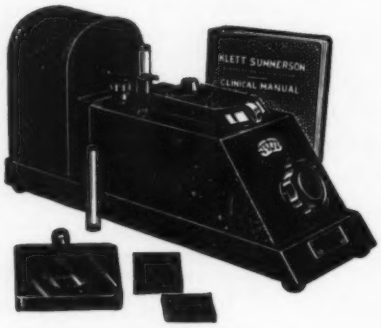
UNI-TECH
Chemical Manufacturing Company
14725 Arminta Street, Panorama City, California
PRODUCTS OF TECHNICAL UNIFORMITY

KLETT SCIENTIFIC PRODUCTS

Klett-Summerson Photoelectric Colorimeters, Colorimeters, Nephelometers, Fluorimeters, Bio-Colorimeters. Comparators, Glass Standards, Glass Cells, Klett Reagents

THE KLETT-SUMMERSON

PHOTOELECTRIC COLORIMETER

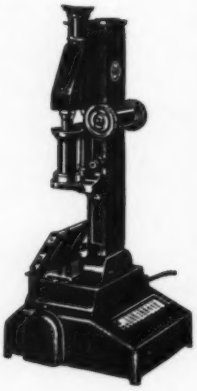


Developed with the requirements of routine colorimetric analysis particularly in mind, but has found application in a variety of unusual analytical procedures as well. No logarithm tables or "calibration curves" on semilogarithmic paper required. The concentration of unknown is obtained directly from the scale reading by simple calculation.

$205

KLETT BIO-COLORIMETER

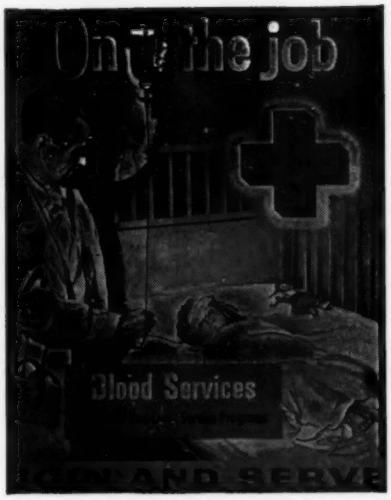
The visual colorimeter illustrated presents a number of outstanding features making it ideal for clinical or research work. Built on the most modern mechanical and optical principles it is widely accepted for its accuracy and durability.



$165

Klett **MANUFACTURING CO.**

179 EAST 87th STREET, NEW YORK, N. Y.



NATIONAL FOUNDATION FOR ASTHMATIC CHILDREN

GIVE

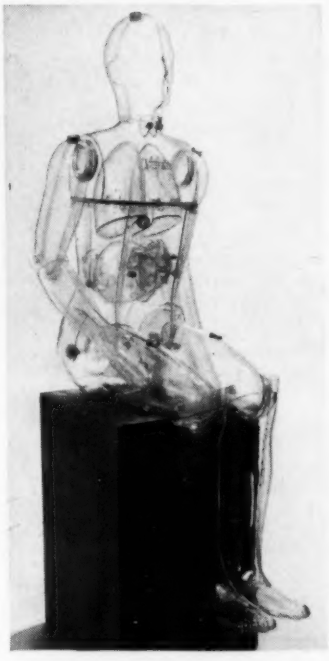
THAT WE MAY BREATHE

NATIONAL HEADQUARTERS • TUCSON, ARIZONA

Kodak reports on:

Tenite Butyrate men . . . a job for the halogen-changer . . . how a clever fellow can cheat himself

A phantom



Dozens of these phantoms have been manufactured out of our *Tenite Butyrate* for government, industry, universities, and hospitals by Alderson Research Laboratories, Inc., 48-14 33rd Street, Long Island City 1, N. Y. We know an interesting phantom when we see one and will forgive those who do not know that the word means a dummy which simulates the human body in studies with ionizing radiation. (In perfecting the new medical x-ray films to make them give the kind of rendition that a radiologist best understands, our phantoms were radiographed so frequently they almost spit fire.) The *Tenite* phantom here shown acts as an emitter for calibrating whole-body counters and such, its Tenite thyroid, liver, lungs, spleen, kidneys, and other organs filled with radioactive solutions that simulate the radiative properties of a radioisotope-treated organ. Alternatively the Tenite shell can be equipped with a human skeleton inside and a system of dosimeter ports for studies of absorption of radioactivity.

How the mind wanders

Methanesulfonyl Fluoride (Eastman 8368) is the result of a day spent listening in on the ACS Division of Medicinal Chemistry and in particular a paper on "Preparation of Some Purinesulfonamides from the Corresponding Purinesulfonylfluorides." We don't recall its content with any precision. What the speaker succeeded in doing to us was to impress us with the strong role being played in biochemical thought today by the sulfonyl group. His mind was doubtless illuminating the big picture, but our mind was narrowing down to the little picture. Here was a trend, and we ought to make sure we were riding it well.

We offer *Methanesulfonic Acid*, *Methanesulfonic Anhydride*, *n-Butyl Methanesulfonate*, *Ethyl-methanesulfonate*, *Methyl Methanesulfonate*, and *Methanesulfonyl Chloride*. This fellow, we noted, was talking sulfonylfluoride. We have a man who is skilled in the art of exchanging any halogen for any other. We decided that as soon as we got home we would have him switch over some chloride to fluoride. That he has done. We are now ready to accept our small but rightful reward for thoughtfulness.

If you want some, or, what is more likely, a look at the list of some 3900 Eastman Organic Chemicals which we stock and which we properly reveal in our Catalog No. 42, get in touch with: Distillation Products Industries, Rochester 3, N. Y. (Division of Eastman Kodak Company).

Mighty magnification

Forty bucks for a few rings and rods to put over the front of the new *Kodak Retina Reflex III Camera?**

A mite under, actually, at the camera shop. Also includes an auxiliary lens. All nicely fitted together. Called the Kodak Retina 1:1 Copying Kit. *Switch the f/2.8 lens from the camera to the kit, snap the kit onto the camera, attach the auxiliary lens and the camera's lens hood, set the diaphragm between f/11 and f/22 and the distance scale at 5 feet. Snap, swish—just like that. Make pictures. Anything in the plane of the rectangular opening is focussed on the film same size. Depth of field at f/22 is 5.5 mm. When resulting slide is projected, magnification is mighty and can be useful for mensuration if a calibrating scale has been included in the picture. Handy for picturing tissue specimens, the triumphant crystals of a new enzyme you have isolated. Kit also includes a slide holder and diffusion screen for making your own black-and-white duplicates of color slides.*

If you can make this kit yourself for less than $40, you are too clever a fellow to be working that cheap.

---

*Most versatile 35mm camera we make. Also works with *Kodak Retina Reflex S* and *IIIS Cameras*. Kit designed for f/2.8 models only.

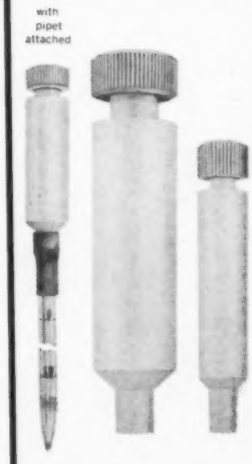
Price is subject to change without notice.

Kodak
TRADE MARK

This is another advertisement where Eastman Kodak Company probes at random for mutual interests and occasionally a little revenue from those whose work has something to do with science

for simple one-hand operation!

"ACCROPET" pipettors



Eliminates dangers and possibilities of contamination inherent in conventional pipetting methods. Compact construction provides simple one-hand operation, giving accurate discharge and filling at great speeds.

Made of high temperature polypropylene, which can withstand temperatures up to 320° F. "O" Ring construction gives highly accurate, positive, leak-proof action. Rubber tubing connection allows great flexibility, which helps eliminate pipet breakage. To operate, attach outlet to pipet directly, with a short length rubber tubing, or a one-hole rubber stopper. Using thumb and first finger, rotate knob up ¾ turn from its deepest position. Place tip of pipet in liquid, and draw up liquid by rotating knob upward.

To accurately control discharge of liquid, rotate knob down into body of Accropet, using thumb and first finger.

Cat. No. M 77992—Accropet, ultra-micro size. For all pipets up to, and including 2/10 cc., and especially recommended for lambda-pettes. Packed singly or in cartons of six.
$3.50 each $16.00 carton

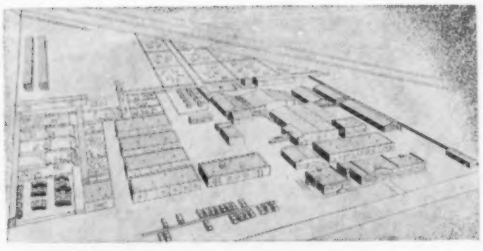
Cat. No. M 77993—Accropet, micro size. For all pipets up to, and including 2 cc., and red and white blood pipets.
$4.95 each $23.70 carton

STANDARD SCIENTIFIC SUPPLY CORP. • 1888 •

STANDARD SCIENTIFIC Supply Corp.

808 BROADWAY
NEW YORK 3, N.Y.

LABORATORY APPARATUS
REAGENTS AND CHEMICALS



Production experience guarantees

RELIABILITY

Order with confidence, the quality and dependability your laboratory and research needs demand. Prompt service. All correspondence and inquiries answered immediately.

- serums
- bloods
- ultrafiltrates
- complement
- globulins
- fluorescent materials
- diagnostic reagents
- tissue culture reagents

We maintain a variety of our own laboratory animals under the finest conditions.

Write for this FREE CATALOG NOW!
No salesman will call.

COLORADO SERUM CO. LABORATORIES

Laboratory and General Office PEAK OF QUALITY

4950 YORK STREET • DENVER 16, COLORADO • MAin 3-5373

FOR MEN AND WOMEN!

A WORTHWHILE CAREER IN LABORATORY TECHNIQUE

THE GRADWOHL SCHOOL OF LABORATORY TECHNIQUE is an ethical school, manned by competent ethical physicians and technologists and enjoys a high rating among the medical profession . . . Graduates placed in desirable positions since 1920. Gradwohl graduates are recognized as expert technicians.

Course includes—Clinical Microscopy; Hematology and Blood Grouping; Serology; Applied Bacteriology; Basal Metabolism; Blood Chemistry; Electrocardiography; Parasitology; Tissue Cutting and Staining.

ENROLL NOW for priority. 12-month course; 6-month internship. Classes begin Jan., March, June, July, Sept., Oct.

40th Successful Year

Write for Free Catalog

GRADWOHL SCHOOL OF LABORATORY TECHNIQUE

Founded in 1920 by R. B. H. Gradwohl, M.D.
Stanley Reitman, M.D., Medical Director
3514 LUCAS AVE. ST. LOUIS 3, MO.

MATTHEWS LIBRARY
A.S.U.

a newly developed colloidal adsorbing agent

Adsormone

for maximum hormone absorption in preparation of urine extracts for pregnancy testing

used by many leading university and government laboratories for fast, accurate tests at lower cost!

INCREASED ACCURACY
RAPID, SIMPLE TECHNIQUE
ELIMINATES DRUG REACTIONS
NO DENATURING OF HORMONE
MORE COMPLETE HORMONE ADSORPTION
NO ANIMAL LOSS FROM TOXIC URINE
COSTS LESS THAN 5c PER TEST
FOR USE WITH FROGS, MICE AND RABBITS

for information write:

LOUIS C. HERRING & CO.
25 EAST UNDERWOOD AVE. ORLANDO, FLA.

ENZYMATIC PREPARATIONS

alpha-CHYMOTRYPSIN 3x cryst, salt-free, lyoph., 8,000 ATEE units/mg	$ 17.00/g
beta-CHYMOTRYPSIN 2x cryst, salt-free	32.50/g
gamma-CHYMOTRYPSIN 2x cryst, salt-free lyoph.	32.50/g
alpha-CHYMOTRYPSINOGEN 6x cryst, salt-free	11.00/g
DESOXYRIBONUCLEASE salt-free, lyoph., 90,000 Dornase units mg	75.00/ 250mg
HYALURONIDASE salt-free, lyoph., 300 Int. units/mg Ovine	22.00/g
salt-free, lyoph., 300 Int. units/mg Bovine	24.00/g
salt-free, lyoph., 500 Int. units/mg Ovine	30.00/g
HYALURONIC ACID lyoph., protein-free	180.00/g
PEROXIDASE salt-free, lyoph., 60 units/mg, RZ = 0.6	55.00/g
RIBONUCLEASE 5x cryst, salt-free, proteose-free, 40 Kunitz units/mg	45.00/g
TRYPSIN 2x cryst, 50% $MgSO_4$, 8,000 + BAEE units/mg	9.00/g
TRYPSINOGEN 1x cryst, 50% $MgSO_4$	12.00/g
URICASE lyoph., hog liver, 5.0 units/g, with $NaCO_3$	18.00/g

Write for Catalog No. 3 Listing Over 12,000 Rare & Fine Organics

K & K LABORATORIES, INC.
177-10 93rd AVE JAMAICA 33, N. Y.

FISKE OSMOMETERS

Available for IMMEDIATE DELIVERY

ACCURATELY MEASURES OSMOTIC PRESSURES by FREEZING POINT METHOD

- Check for overhydration, dehydration, renal shutdown or other kidney disease.
- Measures Hyperosmolarity not detectable by other methods.
- Monitors Osmolarity of parenteral, artificial kidney and dialyzer fluids.
- Provides diagnostic information in diabetes insipidus or mellitis.

FISKE OSMOMETERS, now in use in over 600 research centers and laboratories, prove the reliability of this instrument.

Today's FISKE OSMOMETER, available in laboratory or portable models, offers these . . .

NEW, EXCLUSIVE FEATURES:

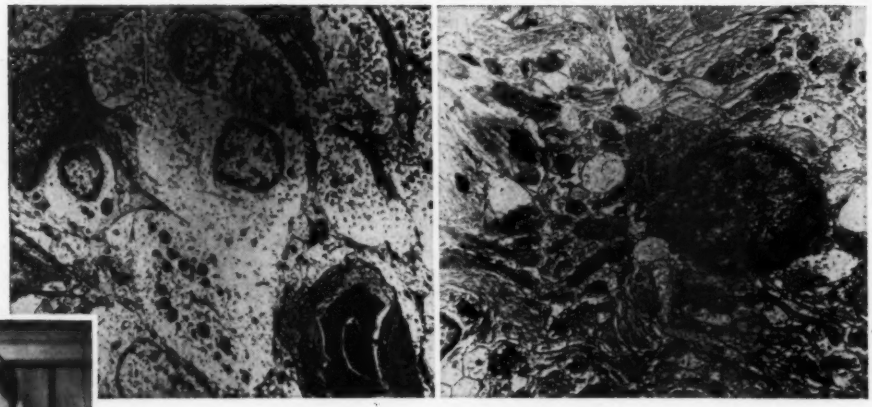
- SHOCK FREEZING
- STAINLESS STEEL UNBREAKABLE TEMP. PROBE
- DUAL STIRRING RODS
- INTEGRATED PRE-CHILL BATH
- ROTARY STIRRING
- BUILT-IN INTERVAL TIMER

For complete details, specifications and applications, write to:

FISKE ASSOCIATES, INC.
BETHEL, CONNECTICUT
Creators of
Precision Electronic Devices

Fig. 1322. Electron micrograph of oligodendroglioma. Several neoplastic cells with abundant pale cytoplasm are arranged near a blood vessel (lower right). Mitochondria are especially prominent in cell at left of blood vessel. (x5,000.) (Courtesy Dr. Sarah Luse.) This illustration appears 40% larger in the book.

Fig. 1325. Electron micrograph. Cellular processes and cells forming this optic nerve glioma are strikingly similar to those of gliosis. From child 6 years of age. (x7,000.) (Courtesy Dr. Sarah Luse.) This illustration appears 40% larger in the book.



One of the most up-to-date, thorough and authoritative books on general pathology in print today

W.A.D. Anderson *New 4th Edition!* **PATHOLOGY**

This new 4th edition of PATHOLOGY is one of the most complete and detailed one-volume works of its kind—encompassing general pathology, special or organ pathology, and surgical pathology. In preparing this revision, W.A.D. Anderson, M.D., has followed the exceptionally successful format of previous editions and again surrounded himself with 34 well qualified pathologists and authors to produce a well rounded book that is authoritative, up-to-date and accurate in every detail.

Virtually every part of the book has been extensively revised, and several areas have been completely rewritten. You will be particularly pleased to find that in this new 4th edition special attention is given to radiation effects, oral and dental diseases, viral diseases, and tropical and parasitic diseases. New chapters have been added on mesenchymal tumors of the soft tissues and hypersensitivity diseases. New findings and newly recognized conditions have been stressed throughout this new edition, and in particular in those areas where additional knowledge is of clinical significance.

With the addition of more than 90 new illustrations, equal in quality to the photographs shown above, this book continues to be one of the most profusely and most effectively illustrated books on this subject. Among other changes which give Dr. Anderson's classic work a truly modern look is the new, larger page size.

Edited by W. A. D. ANDERSON, M.A., M.D., F.A.C.P., F.C.A.P. Written by 35 contributors. Published August, 1961. 4th edition. 1389 pages, 7¼" x 10½", 1385 illustrations, 7 color plates. Price, $18.00.

Order on 30 Day Approval from

The C.V. MOSBY Company

3207 Washington Boulevard, St. Louis 3, Missouri

routine ULTRAMICRO determinations of OSMOTIC CONCENTRATIONS

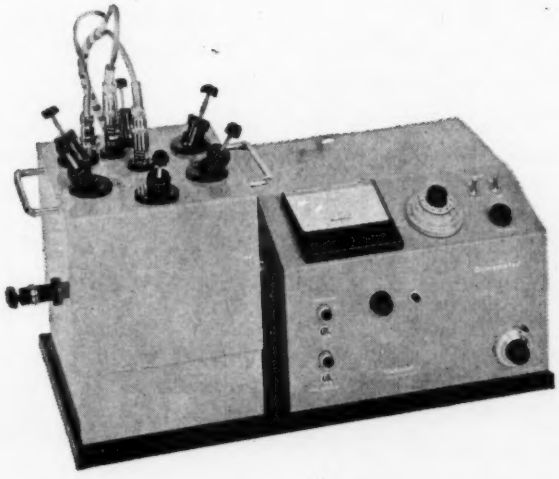
(sample size less than 20 μl)

with the *Mechrolab* Model 301A OSMOMETER

Operates by vapor-pressure lowering—aqueous and non-aqueous solutions—providing accurate determinations within 2 minutes from sample size less than 20 μ-liters. Temperature control to better than 0.001°C. High precision—temperature difference of 0.01°C, read to better than 1%. Simple operation, no installation costs.

Over 150 leading laboratories purchased the Model 301 Osmometer in the past year, and have proved its reliability and accuracy through everyday use.

Mechrolab, Inc. 1062 Linda Vista Avenue, Mountain View 3, California



THE AMERICAN CANCER SOCIETY

is dedicated to saving lives from cancer and spearheads the fight against cancer quackery. Its Committee on New or Unproved Methods of Treatment of Cancer has a membership of physicians, lawyers, educators, and public relations specialists. This committee has been a prime mover in developing constructive action

against cancer quackery

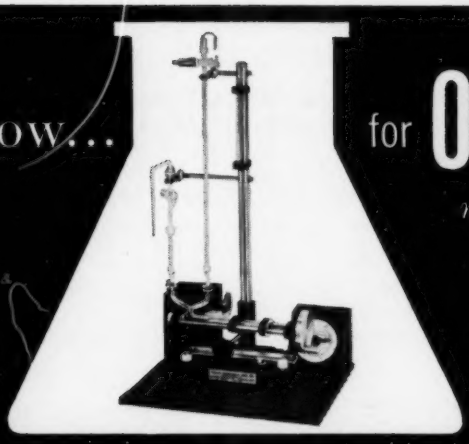
Inspired by model legislation formulated by this committee with the active cooperation of the California Medical Association, California, Kentucky and Nevada recently passed bills providing the first effective means of fighting cancer quackery at its base of operations—in the local community.

To keep both the public and the medical profession informed, the Society has established, in its national office, a central repository of material on new or unproved methods of cancer diagnosis, treatment and cure—a principal source of such information in this country.

The American Cancer Society, in this as in all its efforts, serves both the private citizen and the practicing physician—and is, in turn, served by both.

THE AMERICAN CANCER SOCIETY

Now...



for O_2 and CO_2

more practical and convenient than ever.

MODEL 600

NATELSON MICROGASOMETER with Motorized Automatic Shaker

Thousands of NATELSON MICROGASOMETERS ARE in use throughout the world for blood gas analysis. This growing popularity is based on the advantageous combination of micro specimens with remarkable ease and convenience of use. If you are not now using this popular apparatus you will want full particulars. If you are using the NATELSON MICROGASOMETER you will want the **latest information about accessories** and the new supplement to the instruction booklet **explaining how new reagents and attachments increase the usefulness of this fine instrument.**

Ask your Laboratory Supply Dealer for particulars, or write to:

Scientific Industries Inc.
Dept. LCM-10, 15 Park St., Springfield 3, Massachusetts

Clinical Pathologist Exhibit, Seattle, Wash., Oct. 2-5—Booth 56, Eastern Analytical Symposium, N.Y.C., Nov. 15-17

PHOTOVOLT DENSITOMETRIC EQUIPMENT for ELECTROPHORESIS and CHROMATOGRAPHY

New building-block system permits adding of units as required, from manual and semi-automatic operation to fully-automatic recording and integrating

- **For scanning of electrophoresis strips and readings on large sheets in chromatography**
- **For work in visible and ultraviolet ranges**
- **For evaluation by color-transmission, reflection or fluorescence**
- **For readings on filter paper, agar, starch and other gels**

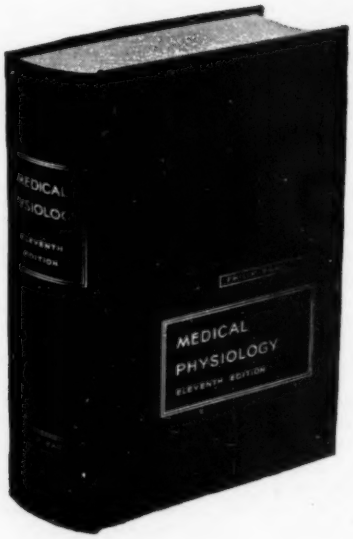
Write for Bulletin 800-S to:
PHOTOVOLT CORPORATION
1115 Broadway • New York 10, N. Y.

A completely redesigned new edition of the finest medical physiology book in the English language

Just Published!
New 11th Edition

MEDICAL PHYSIOLOGY

Edited by Philip Bard
Written by 16 Contributors



Through 10 editions this book has gained the reputation of being the finest textbook in the field of medical physiology, noted particularly for its authoritative writing, its scientific accuracy and its comprehensive, yet practical elucidation of the subject. The new 11th edition of this classic offers a scientific up-to-dateness and completeness unmatched by any other volume in the field.

MEDICAL PHYSIOLOGY is edited and written by 16 of the world's foremost physiologists and educators who are active participants in the dynamic advance of knowledge being made within every sub-specialty of physiology. For example, Mero R. Nocenti, an outstanding leader in the field of endocrinology, has completely rewritten the section on "The Endocrine Glands"; and the well known Christian J. Lambertsen has rewritten the section on "Respiration." In addition, every other section has been revised by equally qualified contributors to give this text an almost newsworthy quality in its review of current physiologic research. Entirely redesigned in a new, larger two-column typographic format, this handsome new edition is profusely illustrated with 70 additional photographs, drawings and charts which clarify the text matter.

This magnificent text is the most comprehensive one-volume work in the field of medical physiology. You'll find no sacrifice of detail or superficial oversimplification for the sake of brevity alone in this book. Its extensive bibliography is probably the most complete and current single source of reference material available.

This textbook can help stimulate your students to study, to think about and to understand medical physiology. The volume selects the material the editor and contributors feel is most significant for the elucidation of each sub-specialty and presents it in terms of the experimental procedures which provided it. No attempt is made to minimize controversial matters or others about which it is necessary to suspend judgment at this time.

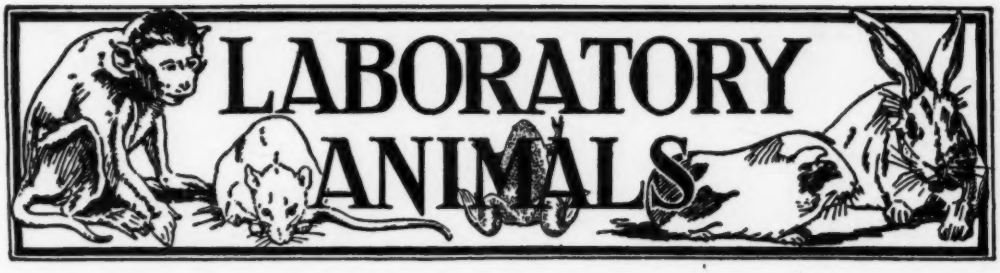
Edited by PHILIP BARD, Professor of Physiology, The Johns Hopkins University. With 15 collaborators. Just published. 11th edition, 1339 pages, 7¼" x 10½", 508 illustrations, 4 in color. Price, $16.50.

CV M

Order on 30 Day Approval From

The C. V. MOSBY Company

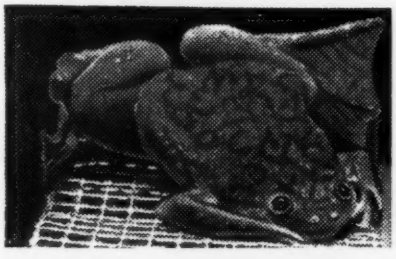
3207 Washington Blvd., St. Louis 3, Mo.



(See also pages 48, 49)

SINCE 1945

XENOPUS LAEVIS FROGS
exclusively



X-L FEMALE FROG PREGNANCY TEST

No Seasonal Variations
No False Positives

Highest accuracy—economy—speed. Test so simple requires minimum of technician's time using either urine concentrate or blood serum. No microscope work or surgery of the animal. Write for literature. Animal cost per test 10¢ to 15¢.

JAY E. COOK, Importer
Cockeysville, Maryland, U.S.A.

RABBITS **GUINEA PIGS**

Developed and housed to insure best results in all types of tests

Continuous supply and prompt shipment FOB Indianapolis

PAUL HAMM RABBITRY R.R. No. 4 TUcker 1-2188 Greenwood, Ind.

Male Frogs for Pregnancy Tests

One dozen $3.50, or $3.00 per dozen for 2 dozen or more. Mealworms (food), thousand $3.35. Postage extra. Frog Bulletin and Scientific Supply Catalog on request.

QUIVIRA SPECIALTIES CO.

4204 W. 21st Street -:- -:- -:- Topeka 32, Kansas

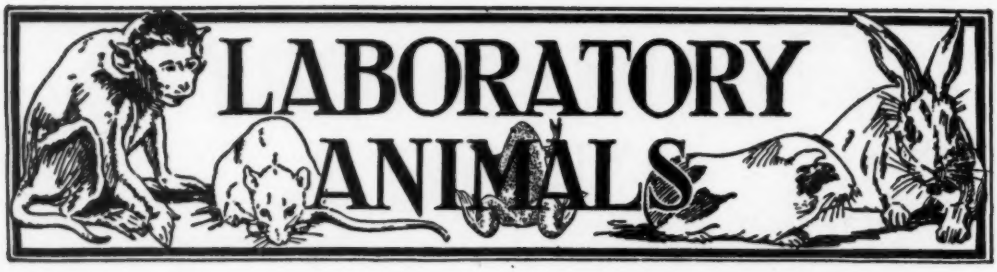
FROGS

Special Males for Pregnancy and Pre-operative Diagnosis.

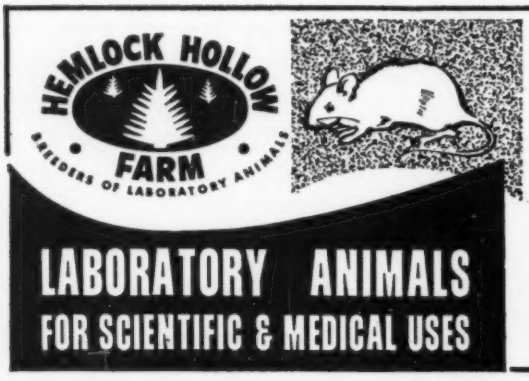
Fast Service. All orders shipped via air same day received. Live delivery guaranteed.

Write for handbook giving full information

E. G. STEINHILBER & CO., INC.
102 Josslyn Avenue (Est. 1922) Oshkosh, Wis.



(See also pages 47, 49)



RATS • • MICE
GUINEA PIGS • • RABBITS
Clean, Ventilated up to date Buildings-
Industrial Washing Machine, Steam
Sterilizer - Regular Testing of Stock
for Detection of Disease.
Send for brochure OX-bow 4-0703
HEMLOCK HOLLOW FARM
414 BLACK OAK RIDGE ROAD WAYNE NEW JERSEY

DAN ROLFSMEYER CO.
ALBINO RATS **SWISS (ALBINO) MICE**
Price List on Request
Route 3, Syene Road, Madison 5, Wis. **PHONE ALpine 6-6149**

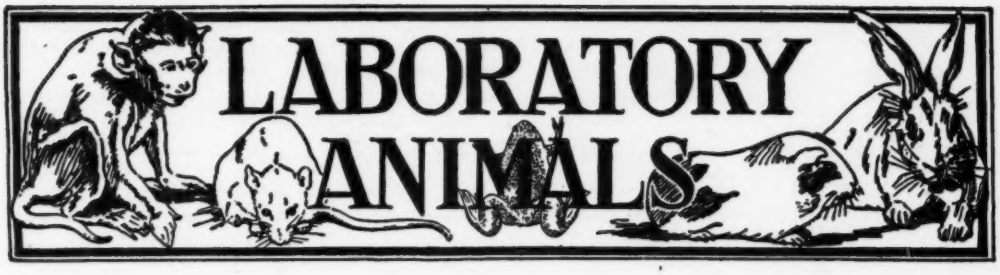
LABORATORY ANIMALS
since 1929

Albino Swiss-W Mice
Albino Rats from Wistar Strain
Albino Guinea Pigs
Albino NZ Rabbits
Hamsters

*stainless steel watering tubes for laboratory animals BENT or STRAIGHT

ALBINO FARMS
P. O. Box 331
RED BANK, NEW JERSEY

We can ship immediately any number of isolated virgin female rabbits in multiples of six or more, suitable for pregnancy tests. Also white males.
Will prepay express or send collect.
CLOVELLY FARM
WILLIAM PRESCOTT (Owner & Director) 6639 Beaver Crest Dr. Lobain, Ohio



(See also pages 47, 48)

LIVE FROGS for PREGNANCY DIAGNOSIS

Rana pipiens

Year 'round supply of selected large, healthy, male frogs for latest pregnancy test (99% accuracy). Cost negligible. Negative test animals can be used repeatedly. Results in less than two hours. Prompt shipment anywhere, by air to distant points. Prices include screened shipping crates and boxes.

Frogs. With complete directions for use and care. Dozen	**$ 7.50**
Laboratory Tank with Cover	**7.00**
Isolation Jar, 5" x 7" with weighted metal cover	**3.50**
Frog pregnancy test kit, complete, 14 items including 12 live frogs	**35.00**

Est. MSSC 1919

NEW YORK SCIENTIFIC SUPPLY CO. - - - - 28 West 30th St., New York 1, N. Y.

Headquarters for Live Frogs

FROGS HORMONE TESTED

Greater accuracy is achieved when you know the sensitivity of the animal.
Free Laboratory Manual on pregnancy testing and the care of frogs in the laboratory.
Be sure of year round supply at lowest prices and fastest service available.
Orders delivered within 36 hours after we receive them.

For information and prices, phone or write:

J. R. SCHETTLE FROG FARM, INC. **Stillwater, Minnesota**

WHITE MICE GUINEA PIGS RABBITS

Swiss-Webster Cross—Very Docile Mouse

"ALL MICE RAISED BY ME ARE RAISED IN PLASTIC PENS"

STOCKER ANIMAL FARM, 44 N. Central Ave., Ramsey, N. J. · Phone: DAvis 7-1064

LABORATORY ANIMALS

We are specializing in an excellent strain of Albino Guinea Pigs—heavy in Hartley Blood Lines. Highly acclaimed by all users. Also Webster Swiss Albino Mice and Wistar Albino Rats.

Dogs	**Rats**	**Rabbits**
Cats	**Pigeons**	**Hamsters**
Mice	**Poultry**	**Guinea Pigs**

JOHN C. LANDIS, Hagerstown, Maryland

REFERRED BIOCHEMICAL ANALYSES

Electrophoresis for Protein Fractionation, Lipoprotein Fractionation, Abnormal Hemoglobins; **Catecholamine** determinations for adrenaline and noradrenaline; **Transaminase** levels; **Hormone** Assays; **Protein-Bound Iodine** determinations; **Toxicological** Assays; Antistreptolysin-O Titers; and other difficult and time-consuming tests.

Services available nationally by Air Mail. Shipping containers with vials available on request

Write for: List of Services, Literature, and Containers

CHEM-TECH LABORATORIES

ANALYTICAL - CLINICAL - RESEARCH

2361/2-2381/2 SOUTH ROBERTSON BLVD., BEVERLY HILLS, CALIFORNIA

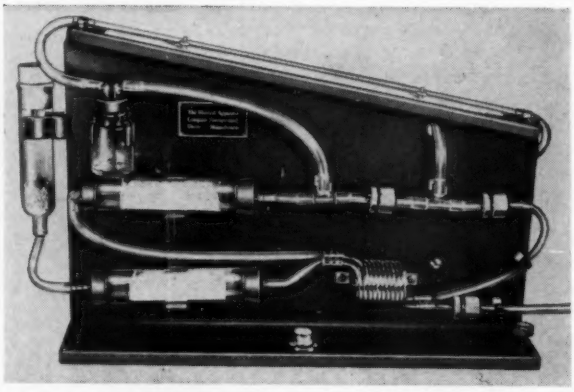
S. I. DULKIN, PH.D. OTTO E. LOBSTEIN, PH.D

CONTINUOUS CO_2 ANALYZER

Catalog No. 2000

This is an inexpensive CO_2 analyzer based on the critical orifice principle.* Its simple construction and ease of operation make it suitable for student use. The unit provides for continuous analysis of both expired gas and alveolar gas (e.g. as provided by Rahn end-tidal sampler). The percentage of CO_2 present is shown by means of a slant manometer. The sampling rate is such that the unit can be used in series with a Pauling oxygen analyzer. Furnished ready for operation including all chemicals.

** J. Mead. A critical orifice CO_2 analyzer for student use. SCIENCE, Vol. 121, pages 103–4, 1955.*



SPECIFICATIONS

- Sample Flow Rate: 100 cc./min.
- Range: 0 to approx. 10% CO_2
- Accuracy: $\pm$ 0.05% CO_2
- Response Time: 2 to 3 minutes for final reading
- Vacuum Requirements: simple water aspirator which reduces water to at least half atmospheric
- Chemicals: Ascarite, Drierite, Gauge Oil

$145.00—f.o.b. Dover, Mass.

Data Sheet and Catalog available on request

HARVARD APPARATUS CO., INC. • Dover, Mass., U. S. A.

(a non-profit organization)

Changing Your Address?

WHEN YOU MOVE, PLEASE—

(1) Notify us to change your address—allow us six weeks to make the change.
(2) Mention the name of this Journal. (We publish twelve periodicals.)
(3) Give us your old address. If possible, return the addressed portion of the envelope in which we sent your last copy.
(4) Give us your new address—complete—including the Postal zone number.
(5) Please print your name and address.

Thank You!

Circulation Department, The C. V. Mosby Company, Publishers, 3207 Washington Blvd., St. Louis 3, Mo.

INDEX TO ADVERTISERS

Accropet
Standard Scientific Supply Corp. ... 40
Acuplastin
Ortho Pharmaceutical Corporation ... 52
Adams Thrombitron
Clay-Adams ... 6
Adsormone
Louis C. Herring & Co. ... 41
AO TS Meter
American Optical Company ... 18
ASTO
Difco Laboratories ... Second Cover
Auto Analyzer
The Technicon Company ... Third Cover

Beta-L Test
Hyland Laboratories ... 13
Biochemical Analyses
Chem-Tech Laboratories ... 50
Biochemicals, Radiochemicals, Pharmaceuticals
Schwarz BioResearch, Inc. ... 17
Blood Gas Analyzing System
Instrumentation Laboratory, Inc. ... 12

Chlori-Pak
Uni-Tech ... 37
Chromatography
Arthur H. Thomas Company ... 36
Clot-Timer
Aloe Scientific ... 2
Coleman, Jr.
Coleman Instruments, Inc. ... 26
Combistix
Ames Company, Inc. ... Fourth Cover
Continuous CO_2 Analyzer
Harvard Apparatus Co., Inc. ... 50
Cytoxan
Mead Johnson Laboratories ... 10, 11

Densitometric Equipment
Photovolt Corporation ... 45
Diagnostic Antigens and Antisera
Cappel Laboratories ... 21

Electron Microscopes
Fisher Scientific ... 22, 23
Enzymatic Preparations
K & K Laboratories, Inc. ... 41

Furoxone Liquid
Eaton Laboratories ... 4

Handi-Boy
Hartman-Leddon Company ... 31

Ketoset
Scientific Products ... 16

Klett-Summerson
Klett Manufacturing Co. ... 38

Lab-aid
The Technicon Company ... 28
Laboratory Animals
Albino Farms ... 48
Clovelly Farm ... 48
Jay E. Cook, Importer ... 47
Paul Hamm Rabbitry ... 47
Hemlock Hollow Farm ... 48
John C. Landis ... 49
New York Scientific Supply Co. ... 49
Quivira Specialties Co. ... 47
Dan Rolfsmeyer Co. ... 48
J. R. Schettle Frog Farm, Inc. ... 49
E. G. Steinhilber & Co., Inc. ... 47
Stocker Animal Farm ... 49
Laboratory Supplies
Harshaw Scientific ... 30
Laboratory Technique
Gradwohl School of Laboratory Technique ... 40
Labware
Corning Glass Works ... 8, 9
Leitz Labolux
E. Leitz, Inc. ... 33

Medotopes
E. R. Squibb & Sons ... 29
Microgas
Scientific Industries ... 45

Osmometer
Mechrolab, Inc. ... 43
Osmometers
Fiske Associates, Inc. ... 41

pH Meter
Beckman Instruments, Inc. ... 25

Reagents and Standards
Paragon C. & C. Co., Inc. ... 34, 35

Sanborn Dual Monitor
Sanborn Company ... 20
Scintillation Detector
Picker Nuclear ... 19
Serums
Colorado Serum Co. ... 40
Simplastin
Warner-Chilcott ... 24
Specialized Analyses
Biochemical Procedures, Inc. ... 27

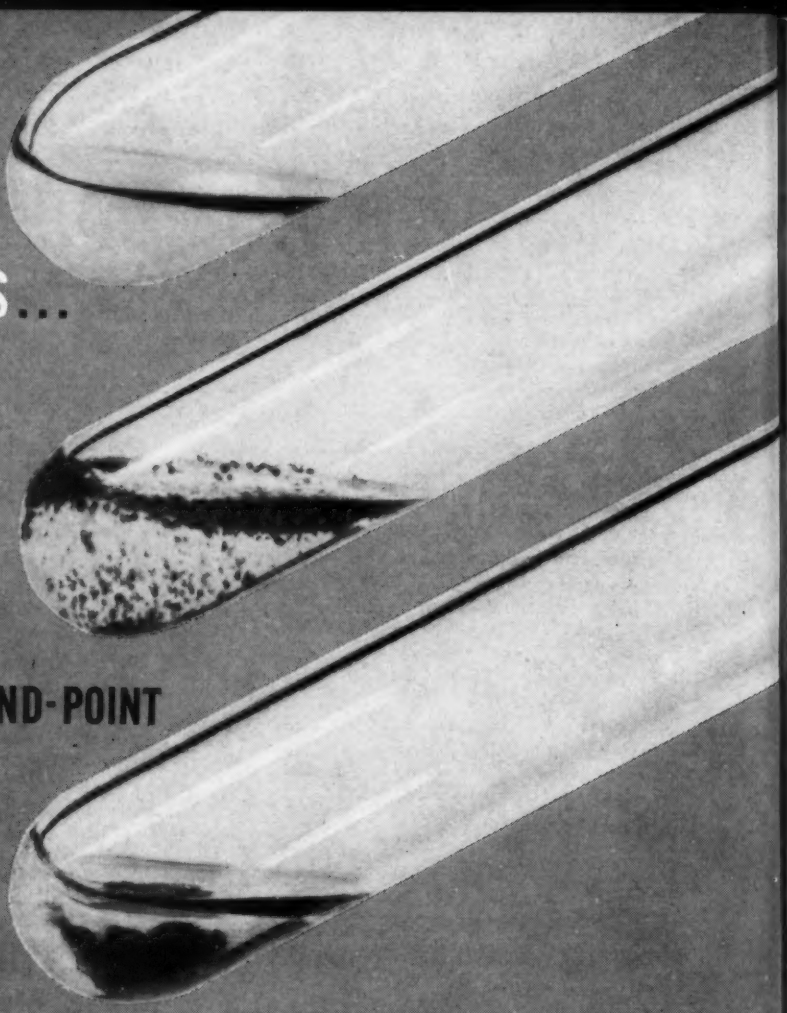
Tenite Butyrate
Eastman Kodak Company ... 39

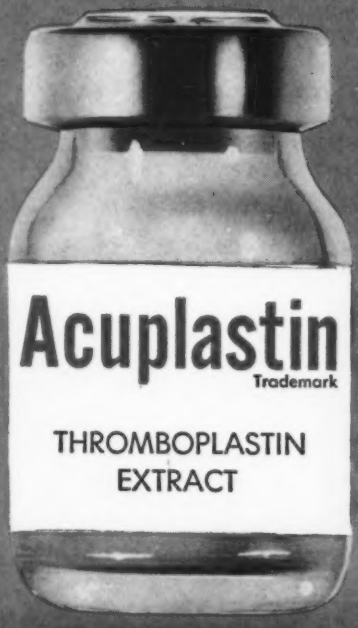
Versatol Pediatric
Warner-Chilcott ... 14, 15

While every precaution is taken to insure accuracy, we cannot guarantee against the possibility of an occasional change or omission in the preparation of this index.

IN PROTHROMBIN TIME DETERMINATIONS... THREE OUTSTANDING ADVANTAGES

- GREATER ACCURACY
- MORE EASILY READ END-POINT
- SENSITIVITY





greater accuracy for more precise clinical control. ACUPLASTIN measures the combined activity of prothrombin *and* proconvertin, an important consideration in anticoagulant therapy.

more easily read end-point—The granular pattern of the formation of fibrin with ACUPLASTIN permits the technologist to observe the formation of a distinct and definite end-point.

sensitivity—ACUPLASTIN detects, and can reproduce small but significant variations in plasma samples.

81458

Ortho Pharmaceutical Corporation
Raritan, New Jersey

Ortho

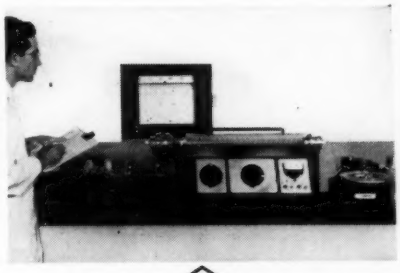
the AutoAnalyzer® Announcer

being intelligence on current developments in the automation of wet chemistry

new squeeze play quadruples tubing life . . . pumps better too

9

AutoAnalyzer users will tell you that for differential metering, the performance of the AutoAnalyzer Proportioning Pump is little short of dazzling. Still, the very vigor (hence certainty) of its peristaltic action has been undeniably hard on tubing . . . a problem we've gone all out to lick. *Now we have the answer* . . . one so disarmingly simple that we're still chuckling over the way it eluded us. It's a pair of parallel guard rails which temper the squeeze on tubing by limiting the excursion of the pump platen. So tamed, the rollers gently, but positively, propel the contained fluids without crushing tubing. This enabled an increase in the spring pressure, and yet . . . life tests run have indicated a dramatic drop in wear and tear on tubes . . . they last up to four times longer. What's more, AutoAnalyzer pumps equipped with these new guard rails now handle tubing as small as 0.010" (0.05 ml.) allowing dilutions down to 100:1. Profit at once by this improvement if you're now operating an AutoAnalyzer. *Your easily-installed conversion kit is waiting for you . . . no charge.* Send for it, *giving serial number of your machine.*



it's quadruplets in electrolyte analysis . . . now our researchers (clever fellows) have come up with a layout that assays individual samples for Na, K, CO_2 and Cl *all at the same time.* It's a heady spread of equipment that does the job . . . one Sampler, two Pumps, a Dialyzer, Flame Photometer, two Colorimeters and four Recorders, all operating with such smooth teamwork that the complete analysis is delivered within five minutes of specimen receipt at the laboratory. Unique performance *wowed* visiting chemists at first showing at recent A.A.C.C. meeting.

10

cholesterol in the news again . . . it's simplified . . . only two months ago this column triumphantly crowed over a tubing so tough it could handle hot sulfuric acid, thereby giving the quietus to the by-pass displacement bottle in cholesterol analyses. Now we've found a way to dispense with the flea mixer and heating unit into the bargain . . . *more acid* flowing faster turns the trick . . . *higher sensitization* makes heat unnecessary.

11

physician, test thyself . . . thousands do so at A.M.A. convention . . . Galen would have popped his eyes to see it . . . were *you* there? A mass screening of 2500 Doctors' blood . . . each tested for cholesterol, transaminase, glucose and uric acid, all done matter-of-factly on an AutoAnalyzer set up for the occasion. Not only that but the whole battery of tests were made *simultaneously* on each single sample, with each result for each constituent recorded separately on four separate recorders. Scheduled by the A.S.C.P. as part of their impressive A.M.A. health program, the tests will ultimately ground a statistical survey of normal values. *Impressive vote of confidence in AutoAnalyzer accuracy . . . don't you think?*

12

flame photometer fuel problems . . . here's a way out . . . maybe . . . no longer restricted to propane, the new AutoAnalyzer Photometer burner can handle equally well acetylene, natural gas, butane, what have you? With *that* kind of versatility, it may ease possible local restrictions.

13

Kjeldahl (other nitrogen determinations, too) finally bow to automation . . . by solving the primary *digestion* problem, the new AutoAnalyzer continuous-digestion module succeeds in automating the hard-to-crack Kjeldahl test. Entering the digestor, the nitrogenous sample and appropriate digestion fluid feed into a rotating helical conveyor which propels the tumbling mixture through a high/low heated chamber where oxidation and digestion take place. The clear digestate then passes on to an AutoAnalyzer system where an indophenol blue reaction measures the ammonium sulfate end product.

14

Not only Kjeldahls, but similar digestion requiring tests are opened to automation through this new modality.

plug-in plattered manifolds speed AutoAnalyzer set-up . . . we're now delivering test manifolds completely assembled on a stainless steel *platter* . . . all tubing connections pre-sealed, all glass fittings permanently mounted *ready-to-go.* Makes short work of changeover from one test to the next: simply detach one platter and attach the new one to the machine. As neat and natty a package as you've ever seen: *easy to handle, easy to store, quick to set up.*

15

free calcium — significant diagnostic tool . . . original experiments, employing a modification of the AutoAnalyzer ammonium purpurate murexide technique, point the way to accurate differential assay of free or ionizable calcium and total calcium. Measurement of the small *free* fraction of the total calcium proves to be highly accurate and dependably reproducible. The whole matter is the subject of a recent Ph.D. thesis which reports on the clinical significance of free calcium as a diagnostic tool.

16

TECHNICON INSTRUMENTS CORPORATION
111 Research Park, Chauncey, N. Y.

Gentlemen:

Please send me the TECHNICON AUTOANALYZER BROCHURE plus data on the items checked.

☐ 9 ☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14 ☐ 15 ☐ 16

NAME _____

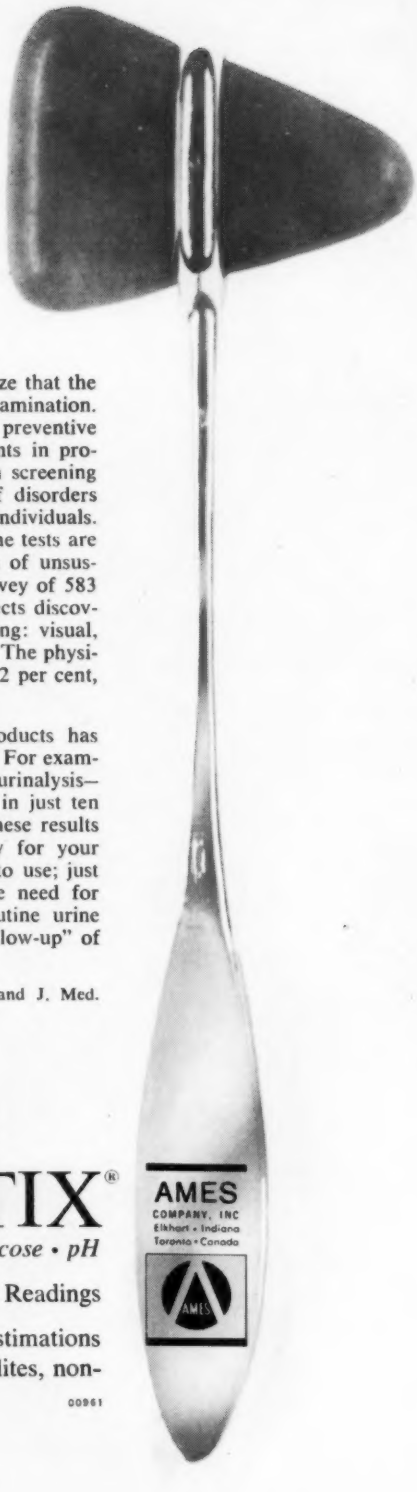
INSTITUTION _____

ADDRESS _____

CITY _____ ZONE _____ STATE _____

AN AMES CLINIQUICK®
CLINICAL BRIEFS FOR MODERN PRACTICE

health screening... vital aid for early diagnosis



In recent years, the patient has come to realize that the best health insurance is a periodic health examination. Since the ultimate goal of medical science is preventive medicine, the benefits of current developments in prophylaxis are undisputed. The value of health screening is indicated by the significant percentage of disorders found in apparently well and asymptomatic individuals. To augment your physical examination, routine tests are of decided value, leading often to diagnosis of unsuspected abnormalities. One Group Health Survey of 583 patients revealed that 49 per cent of the defects discovered were initially detected by technical testing: visual, hearing, x-ray, blood and urine examinations. The physical and the history yielded 39 per cent and 12 per cent, respectively.*

The development of modern diagnostic products has greatly simplified the laboratory examination. For example, three important test results in the routine urinalysis—protein, glucose and pH – now can be had in just ten seconds with COMBISTIX® Reagent Strips. These results are provided accurately and simultaneously for your immediate evaluation. COMBISTIX is simple to use; just "dip-and-read." It completely eliminates the need for elaborate equipment. COMBISTIX makes routine urine testing feasible for health screening and "follow-up" of progress, at minimal cost.

*McCombs, R. P., and Finn, J. J., Jr.: New England J. Med. *248*:165 (Jan. 29) 1953.

for 3 simultaneous test results...

DIP AND READ

COMBISTIX®

urine protein • glucose • pH

1 Dip . . . 10 Seconds . . . 3 Readings

- standardized color chart for reliable estimations
- unaffected by turbidity, drug metabolites, non-glucose reducing substances

00961

AMES
COMPANY, INC
Elkhart • Indiana
Toronto • Canada